THE RELATIONSHIP OF NICOTINIC ACID, TRYPTOPHANE AND PROTEIN IN THE NUTRITION OF THE PIG [1]

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The effects of nicotinic acid deficiency in swine have been well established by Chick and co-workers ('38), Hughes ('43), Madison, Miller and Kieth ('39) and Wintrobe ('39). Recently, Wintrobe and co-workers ('45) showed that when the level of casein in a purified ration was 26%, the absence of nicotinic acid in the ration caused no apparent sign of nutritional deficiency except that of a slightly lowered growth rate. They also showed that when the level of casein in the diet was lowered to 10% and nicotinic acid excluded from the vitamin supplement, typical symptoms of nicotinic acid deficiency resulted. The relationship between protein and nicotinic acid may be explained by the work of Krehl et al. ('45, '46). These workers found that the inclusion of corn grits in a nicotinic acid-low ration caused a marked growth retarding effect when fed to rats. This effect was counteracted by supplements of either nicotinic acid or l(−) tryptophane. When either rice, oats or rye were substituted for corn, good growth was obtained which seemed to indicate that the low nicotinic acid content of corn was not the only limiting factor in its growth retarding effect. Spector and Mitchell ('46), in an application of the paired feeding technique, found that

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the growth-depressing effect of corn on rats fed a nicotinic acid-low ration was associated with a decreased food consumption. These authors further found that the stimulation in growth produced by supplementing the corn grits ration with nicotinic acid or tryptophane was largely, but not entirely, associated with an increased caloric intake.

In view of the fact that corn constitutes a major portion of the ration of swine, particularly in the corn belt states, it seemed advisable to study the possible relationships between nicotinic acid and tryptophane in a ration largely made up of corn.

EXPERIMENTAL

Pigs used in this experiment were farrowed by Duroc Jersey sows which had been bred to a Yorkshire boar. Up to the time of weaning all animals had been kept on alfalfa pasture to prevent, as far as possible, the appearance of any known deficiency. Two weeks before the beginning of the experiment the pigs were vaccinated for hog cholera using the serum and virus method. The three basal rations used are shown in table 1. The protein content of rations A, B and C were 19.2, 14.0 and 15.9%, respectively, and the nicotinic acid contents

TABLE 1

Composition of basal ration.[1]

	RATION A	RATION B	RATION C
	%	%	%
Corn	80	87	
Oats			90
Casein (commercial)	12	5.5	4
Soybean oil meal	6	5.5	4
Complex mineral mixture[2]	2	2	2

[1] The following amounts of vitamins were supplied daily to each animal: thiamine, 10 mg; riboflavin, 10 mg; calcium pantothenate, 25 mg; and pyridoxine hydrochloride, 6 mg.

[2] This mineral mixture contained the following (in %): bonemeal, 32.3; ground limestone, 32.3; sodium chloride, 32.3; ferrous sulfate, 2.5; copper sulfate, 0.2; manganese sulfate, 0.1; zinc oxide, 0.1; cobaltous acetate, 0.1; and potassium iodide, 0.1.

were 5.22, 6.23 and 6.25 mg per pound, respectively, as determined microbiologically by the method of Krehl, Strong and Elvehjem ('43). The higher nicotinic acid content of ration B was due to the fact that a new shipment of corn was used which contained appreciably larger amounts of this vitamin. The 3 rations were supplemented with a vitamin A and D oil in amounts which supplied 1500 I.U. of vitamin A and 200 I.U. of vitamin D per pound of feed. All lots were fed ad libitum, and each pig received daily the amounts of thiamine, riboflavin, calcium pantothenate and pyridoxine hydrochloride indicated in table 1.

Both at the beginning and again during the last week of the experiment the pigs were placed in metabolism cages and a 24-hour urine collection was obtained from each animal. The daily excretion of thiamine, riboflavin, pantothenic acid, nicotinic acid and N^1-methylnicotinamide was determined. In addition the daily excretion of tryptophane was determined in lots 3, 4, 5 and 6. Thiamine in the urine was determined by the method of Mickelsen, Condiff and Keys ('45). Microbiological methods were used for the determination of riboflavin (Snell and Strong, '39), pantothenic acid (Neal and Strong, '43) and nicotinic acid (Krehl et al., '43). N^1-methylnicotinamide was determined by the method of Huff, Perlzweig and Tilden ('45). The assay for tryptophane in the urine was similar to that used by Schweigert, Säuberlich and Elvehjem ('45) who modified the nicotinic acid method of Krehl et al. ('43) by omitting tryptophane from the media and replacing it with nicotinic acid.

The animals were bled at regular intervals and a study made of the cellular constituents of the blood. At the conclusion of the experiment the animals were autopsied and a pathological study made of the affected tissues.

RESULTS

The effect of high protein

Pigs in lot 1 were fed the high protein basal ration A plus daily supplements of 30 mg of nicotinic acid. These animals

gained an average of 1.40 pounds daily as shown in table 2. This represents a higher average daily gain than was shown by the pigs in any other lot in the experiment. In addition the gains for these 5 pigs were more uniform, and all of the animals had the healthy appearance of normal pigs. The animals in this lot consumed an average of 48 mg of nicotinic acid per day. Table 3 shows that the total daily excretion of nicotinic acid plus its metabolic N^1-methylnicotinamide was 25.26 mg per pig or about 53% of the amount ingested. Autopsy of the pigs in lot 1 revealed no gross pathology.

TABLE 2

Response of pigs fed the various experimental rations.

CATEGORY OF INTEREST	LOT NUMBER					
	1	2	3	4	5	6
Ration fed	A	A	B	B	B	C
Number of pigs	5	5	5	5	5	5
Initial age in weeks	8	8	6	6	6	6
Number of weeks on trial	8	8	6	6	6	6
Average initial weight in lbs.	40	39	28	27	27	28
Average daily gain in lbs.	1.40	1.00	0.76	0.62	1.00	0.76
Average daily feed consumption, lbs.	3.64	3.09	2.27	1.98	2.29	2.29
Lbs. of feed per lb. of gain	2.61	3.09	2.98	3.19	2.29	3.01

Pigs in lot 2 were fed ration A. The animals in this lot did not appear to be as healthy as those in lot 1 and their average daily gain was smaller (table 2). Three of the animals in this lot developed a mild diarrhea within 4 weeks. The average daily excretion of N^1-methylnicotinamide was 12.21 mg per pig as compared with 22.34 mg for the animals in lot 1.

From an inspection of both the growth and excretion data the animals did not seem to be as abnormal as one would expect. The autopsy results, however, indicated an abnormal condition in the large intestine. When the abdomen was opened the large intestine appeared thickened and upon palpation felt doughy. The lesions were limited to the large intestine and particularly to that portion of the gut between the cecum and rectum. In some cases the congestion of the

mucus membrane was severe. A yellowish caseated material was firmly adherent to the mucus membrane. Small masses of feces were found adhering to the gut in the affected areas. All other organs studied appeared normal. Thus, although the protein in the ration was high the absence of nicotinic acid resulted in deficiency symptoms which were confined entirely to the large intestine. The fact that Wintrobe et al. ('45) reported no such deficiency when a nicotinic acid-low purified ration was fed suggests that corn plays an important role in the etiology of this deficiency. This further suggests that the relation between corn, nicotinic acid and tryptophane may be similar to that found for the rat (Krehl et al., '46).

The effect of low protein

Ration B containing 14% protein was fed to the pigs in lot 3. Each animal in this lot received 30 mg of nicotinic acid per day. As was expected the animals in this lot were normal in appearance although their growth rate was not as great as that of the animals in lots 1 and 2; however, some of this difference is due to the fact that the pigs in this lot were placed on the experiment at an earlier age and weighed less than the animals in lots 1 and 2 (table 2). The average daily excretion of N^1-methylnicotinamide was 15.13 mg per pig for the animals in this lot. Autopsy revealed no abnormalities of any kind, the gut being perfectly normal.

Pigs in lot 4 were fed the low protein ration B. During the fourth week of the experiment 4 of the animals in this lot developed severe diarrhea. The hair coat appeared rough and the skin around the ears was scaly. Autopsy revealed intestinal lesions similar to those described for animals in lot 2 except that they were even more severe. In some cases the colon had become so weakened that even moderate pressure would tear the walls. No pathogenic bacteria could be isolated from the intestines of these animals. The daily excretion of N^1-methylnicotinamide dropped to an average of 4.92 mg per pig. The symptoms manifested by these animals corre-

spond to those of nicotinic acid deficiency in pigs as described by other workers.

The animals in lot 4 did not develop anemia as evidenced by an average hemoglobin content of 12.55 gm per 100 ml, but a mild leucocytosis was noted. No other morphological abnormalities were observed.

Pigs in lot 5 were fed basal ration B plus daily supplements of 200 mg of d,l-tryptophane. It was evident after the first 2 weeks of the experiment that the animals in this lot were gaining weight more efficiently than pigs in the other lots. The pigs had the sleek healthy appearance of normal animals. As shown in table 2 the average daily gain was 1 lb. per pig and only 2.29 lbs. of feed were required per pound of gain. As was expected the tryptophane excretion was higher than that of lots 3 and 4, but it was surprising to find that the average daily excretion of N^1-methylnicotinamide was 14.00 mg as compared with 4.92 mg for animals in lot 4 (table 3) since neither lot received any added nicotinic acid. Animals in lot 5 consumed 2.29 lbs. of basal ration per day which furnished 14.27 mg of nicotinic acid. The total daily excretion of nicotinic acid and N^1-methylnicotinamide averaged 15.69 mg per pig, which is in excess of the amount ingested. It would thus appear that the animals in this lot were obtaining an additional source of nicotinic acid which is in some way related to the feeding of tryptophane. No corresponding increase in the excretion of nicotinic acid was noted. Rosen, Huff and Perlzweig ('46) have reported that when rats were fed 50 mg of either l-tryptophane or its racemic mixture, an increase in both N^1-methylnicotinamide and nicotinic acid excretion occurred. It is possible that if larger amounts of tryptophane had been fed to the pigs the excretion of nicotinic acid would have been increased. Autopsy of the animals in this lot revealed that in 2 of the pigs a few gross lesions in the colon were observed. Although these lesions were of a minor nature and seemed to have no visible effect on the health of the pigs, it would seem that either the level of tryptophane fed was not sufficient to compensate completely for the lack of nicotinic

acid or that a source of nicotinic acid is indispensable no matter what the level of tryptophane in the diet may be.

Pigs in lot 6 were fed basal ration C made up largely of whole oats, a grain which is also low in nicotinic acid. The level of protein was 15.9% as compared with 14.0% in ration B. The growth response of these pigs was superior to that of the animals in lot 4 which had been fed the low protein corn

TABLE 3

Average daily urinary excretion of B vitamins, tryptophane and N^1-methylnicotinamide.[1]

LOT NO.	THIAMINE	RIBOFLAVIN	PANTOTHENIC ACID	NICOTINIC ACID	N^1-METHYL-NICOTINAMIDE	TRYPTOPHANE
1	1.68 [2]	2.70	14.25	2.92	22.34	.....
	0.40–2.50 [3]	1.05–3.42	9.63–18.00	1.92–4.51	19.10–31.81	
2	1.33	1.83	19.22	1.91	12.21	.....
	0.81–1.82	1.53–2.12	13.04–25.52	0.94–2.75	7.25–16.30	
3	1.86	1.72	10.19	2.28	15.13	14.25
	1.00–8.27	1.04–2.67	8.25–13.60	1.50–3.50	14.50–19.86	9.01–17.00
4	1.30	1.45	14.38	1.31	4.92	10.71
	1.02–1.65	1.11–2.16	12.43–18.06	1.07–1.57	3.46– 7.64	8.05–14.00
5	1.27	1.59	11.54	1.69	14.00	20.94
	0.78–2.00	1.04–2.63	7.80–14.56	1.08–2.31	12.32–16.03	19.00–24.21
6	5.48	1.78	17.51	1.34	8.98	19.75
	2.67–7.71	1.20–2.32	14.60–25.00	1.05–1.48	8.40– 9.61	16.01–25.10

[1] All data are in milligrams per 24 hours.
[2] Average.
[3] Range.

ration. The pigs developed a rough hair coat and were untidy in appearance but did not exhibit diarrhea. The excretion of tryptophane was almost as high as that of the animals in lot 5 which received tryptophane. The pigs in this lot ingested 13.91 mg of nicotinic acid daily and excreted 8.98 mg of N^1-methylnicotinamide. There were no intestinal lesions found at autopsy but the colons of all animals were distended with oat hulls. The poor growth rates of the pigs in this lot may be explained by the high fiber content of the ration.

Urinary excretion of thiamine, riboflavin and pantothenic acid

The daily excretion of thiamine, riboflavin and pantothenic acid was obtained from all of the experimental pigs. It is evident from table 3 that only a small proportion of the additional thiamine and riboflavin fed to the pigs was excreted in the urine. The higher average excretion of thiamine in lot 6 may be due to the fact that oats, in general, contain considerably more thiamine than does corn. It is interesting to note that in every lot the daily excretions of pantothenic acid were higher than those of the other 2 vitamins.

DISCUSSION

The results indicate that when corn constitutes a major part of the ration nicotinic acid deficiency occurs even though the percentage of protein is relatively high. Wintrobe et al. ('45) were not able to produce nicotinic acid deficiency in swine on a diet containing 26% casein, but when the casein content was lowered to 10% nicotinic acid deficiency appeared. In the present experiment it must be remembered that while the protein content of ration A was 19.2%, it contained only 12% of crude casein and for that reason undoubtedly did not contain as much tryptophane as did the high protein ration of Wintrobe et al. ('45). It is probable that had the percentage of casein in the high protein ration been increased, deficiency symptoms would not have appeared. This is borne out by the fact that the intestinal lesions of nicotinic acid deficiency produced in pigs fed the high protein ration were not as severe as those produced when the low protein ration was fed.

It has been shown by Hughes and Ittner ('42) and by Wintrobe et al. ('43) that a lack of pantothenic acid in the diet of the young pig causes marked inflammation of the intestinal tract and diarrhea. The similarity between the lesions produced in pantothenic acid deficiency and nicotinic acid deficiency have been noted. It does not seem likely, however, that pantothenic acid was a factor in the deficiency syndrome

produced in this experiment. The presence of pathogenic organisms in the intestines of the deficient pigs was eliminated as a factor since bacterological examination of sections of the inflamed colons did not reveal the presence of any of these types of organisms.

The supplementation of the low protein corn ration with tryptophane produced animals whose external appearance was normal in every respect. The animals gained an average of 1 lb. of body weight per day. They also utilized their feed more efficiently than pigs in the other lots. The fact that 2 pigs in this lot did have a mild inflammatory condition of the colon may indicate that a dietary source of nicotinic acid is still necessary even though the ration is supplemented with tryptophane. However, one might also reason that had the level of tryptophane in the ration been higher no such condition would have appeared. The fact that the inclusion of tryptophane in the ration does lead to an increase in the excretion of N^1-methylnicotinamide tends to support the latter view. The exact nature of the relationship between nicotinic acid and tryptophane is not clear. Spector and Mitchell ('46) have postulated that the increase in the excretion of nicotinic acid and N^1-methylnicotinamide following the ingestion of tryptophane indicates that tryptophane may exert a sparing action on the dietary requirement for nicotinic acid, and furthermore that the interrelationship between nicotinic acid and tryptophane may be analogous to the metabolic interrelation of choline and methionine.

The results obtained by feeding a ration largely composed of oats to young pigs seem to be overshadowed by the high fiber content of the ration. It is significant, however, that despite the low nicotinic acid content of ration C no abnormal condition was found in the intestinal tract of these pigs which probably was due to the fact that the oat protein contains considerably more tryptophane than does the protein of corn (Block, '45).

Using a purified ration the daily nicotinic acid requirement of the young pig has been estimated (Hughes, '43) to lie be-

tween 5 and 10 mg per 100 lbs. The results of the present experiment indicate that with corn as the major constituent of the ration the requirement for this vitamin may be higher.

SUMMARY

When a high protein, low nicotinic acid, corn ration was fed to weanling pigs, mild symptoms of nicotinic acid deficiency occurred. The symptoms of nicotinic acid deficiency were confined entirely to the large intestine and particularly to the colon.

The lowering of the protein content of the same corn ration to 14.0% produced very severe inflammation in the large intestine. Supplementing this ration with 200 mg of d,l-tryptophane per day per pig gave excellent growth response in the animal. Two of the animals in this lot showed a very mild inflammatory condition of the large intestine.

No symptoms of nicotinic acid deficiency were noted when the corn was replaced with oats.

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RIBOFLAVIN DEFICIENCY IN THE DAIRY CALF

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FOUR FIGURES

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Evidence (see reviews by Goss ('43) and by Najjar and Barrett ('45)) indicates that the adult ruminant does not require the B vitamins in its diet. Extensive synthesis of the B vitamins occurs in the rumen of the adult bovine; however, it has not been established as to which, if any, of the B vitamins would be required were rumen synthesis not taking place. In order to examine this question the young ruminant in which the rumen is not yet functioning was chosen for experimental study.

The synthesis of riboflavin in the rumen of both the sheep and the bovine has been adequately demonstrated by the work of McElroy and Goss ('39, '40); Wegner et al. ('40, '41) and Hunt et al. ('41, '43). In our work the requirement of the young dairy calf for riboflavin under conditions which would tend to eliminate rumen synthesis has been investigated.

EXPERIMENTAL

The experimental animals were 2 male Guernsey calves and 1 male Holstein calf. The animals, 48 hours old or less when obtained, had been receiving colostrum. They were kept in individual metal cages 5×6 feet in size, and equipped with

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heavy wire mesh bottoms. The experimental diet was a "synthetic milk" having the composition shown in table 1 and containing no riboflavin. The lard and wheat germ oil were homogenized with the solution of casein, cerelose and salts to produce an emulsion containing 13% solids. For complete details concerning preparation of this diet see Wiese, Johnson, Mitchell and Nevens (in press).

TABLE 1

Composition of "synthetic milk" diet.[1]

COMPONENT[1]	PER CENT	VITAMINS ADDED	MG PER KG OF LIQUID DIET
Casein, "Labco"	30.0	Thiamine	0.65
Lard	26.3	Pyridoxine	0.65
Wheat germ oil	0.3	Nicotinic acid	2.60
Salts	4.0	Calcium pantothenate	1.30
Cerelose	39.4	Inositol	26.0
		Choline	260.0
		p-Aminobenzoic acid	2.60
		Pteroyl-glutamic acid ("folic acid")	0.052
		Biotin	0.01
		2-Methyl-1, 4-naphthoquinone	0.26
		Vitamin A	5000 I.U. per day
		Vitamin D	500 I.U. per day

[1] The lard and wheat germ oil are homogenized with the solution of casein, cerelose and salts to produce an emulsion containing 13% solids. For complete details see Wiese, Johnson, Mitchell and Nevens, J. Dairy Sci. (in press).

After the calves had been on the experiment for approximately 2 weeks, the gingival surface of the cheeks and the mucosa of the tongue became hyperemic and the gums bled on applying slight pressure. Lesions were observed at the oral canthi and along the edges of the lips. These observed pathological changes are similar to the cheilosis which has been described as occurring in human riboflavin deficiency.

The condition of the calves became progressively worse. They exhibited a copious, tenacious, chalky-colored salivary secretion which was not shown by calves on other diets. In addition to an excessive and abnormal salivary secretion, ex-

cessive lachrymation was a characteristic symptom. The gains in weight were irregular because of a variable appetite. The hair appeared rough and dull and the animals shed excessively. Certain areas of the body, such as the abdomen, became practically devoid of hair. The calves scoured and the navel region became inflamed and irritated. The photographs of calf no. 14, figures 2 and 3, illustrate the appearance of dairy calves when deficient in riboflavin. The excessive lachrymation and salivation are evident in both photographs, while figure 3 shows loss of hair on the abdomen and inflammation of the navel region.

After 5 weeks on the diet, calf no. 14 was given 5 mg of riboflavin by intravenous injection. On the day following the injection the animal showed an improved appetite and decreased salivation. This improvement was only transitory as the animal again exhibited anorexia and showed excessive salivation on the second day following the injection.

Since the condition of the animal became critical, the diet was supplemented with 5 mg of riboflavin per day. The calf was kept on this supplemented diet until taken off the experiment. Within 3 days after supplementation was begun the animal's appetite improved, the hyperemia of the buccal mucosa disappeared, excessive salivation stopped, and he started to gain weight. In approximately 10 days the lesions in the corners of the mouth, along the edges of the lips and around the navel had healed. New hair began to grow in the areas from which hair had been lost. The immediate and rapid growth response in body weight can be readily seen from figure 1. The improved appearance of the animal after riboflavin administration is shown in figure 4. The lack of secretion from the eyes and mouth is evident when comparison is made with figures 2 and 3.

Calf no. 13 was given 5 mg of riboflavin per day after 6 weeks on the deficient diet. The response to riboflavin administration was the same as that of calf no. 14. The growth data for calf no. 13 are also summarized in figure 1.

Calf no. 15, which was on the deficient diet for 4 weeks only, showed the same symptoms as those reported for calves nos. 13 and 14.

During the course of the experiment periodic examinations of the eye were made with the aid on an opthalmoscope. Neither vascularization of the cornea nor opacity of the lens was observed.

At weekly intervals 24-hour urine collections were made and the riboflavin content determined by the method of Najjar ('41). The data are given in table 2. The first collection was

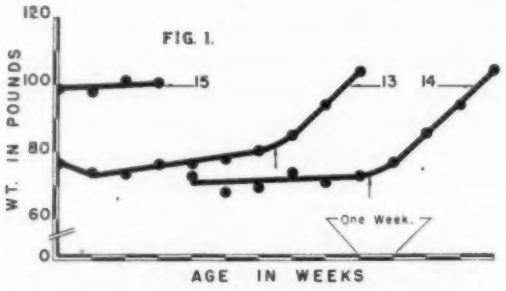

Fig. 1 Growth of calves on riboflavin deficient diet. Calves nos. 13 and 14, Guernseys; calf no. 15, Holstein. The arrow indicates when feeding of 5 mg of riboflavin per day in diet began.

made when the animals were 48 hours old and had received colostrum. Under these conditions the amount of riboflavin excreted was high. After 1 week on the riboflavin-deficient diet there was a marked drop in the amount of riboflavin excreted and by the third week little or no riboflavin was found in the urine. When riboflavin was added to the diet, however, there was an immediate rise in the urinary riboflavin excretion. The practical disappearance of riboflavin from the urine of the calves on a diet devoid of this vitamin suggests strongly that synthesis of the vitamin was not occurring, either in the intestinal tract or in the tissues. These results are in contrast to those secured with nicotinic acid, a vitamin not necessary in dairy calf nutrition. On a ration free of this

Figs. 2 and 3 Calf no. 14 after being on riboflavin deficient diet for 5 weeks. Note excessive salivation and lachrymation. Figure 3 shows loss of hair on abdomen and inflammation around navel.

Fig. 4 Calf no. 14 after receiving riboflavin supplement for 3 weeks. Note absence of symptoms.

vitamin, the urinary excretion is quite constant and of considerable magnitude (Johnson et al., in press).

When the animals were sacrificed at the close of the experiment, postmortem examination failed to reveal any internal gross lesions. Sections from the brachial and sciatic nerves, stained with hematoxylin-eosin and Mahon's technique, did

TABLE 2

The urinary excretion of riboflavin.

WEEKS ON DIET	CALF 13		CALF 14		CALF 15	
	Riboflavin fed	Riboflavin excreted	Riboflavin fed	Riboflavin excreted	Riboflavin fed	Riboflavin excreted
	mg/day	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*
0	0	7.59	0	10.59	0	1.16
1	0	0.08	0	0.09	0	0.02
2	0	0.040	0	0.01	0	0.07
3	0	...	0	0.06	0	...
4	0	0.06	0	0.08	0	0.06
5	0	0.0	5	0.78	0	0.17
6	0	0.0	5	1.38		
7	5	...	5	...		
8	5	0.38	5	1.44		

not show any microscopic lesions. The fresh weights of the rumen-reticulum for calves 13, 14 and 15 were, respectively, 381, 356 and 349 gm. as compared with weights of 376, 407 and 360 gm for the omasum-abomasum. These measurements reveal the undeveloped condition of the rumen.

DISCUSSION

The young dairy calf fed a "synthetic milk" diet requires riboflavin. The low amounts of riboflavin excreted in the urine on a diet devoid of this vitamin indicate that appreciable synthesis of the riboflavin in the rumen or in the tissue did not occur. Furthermore, the weight of the rumen and reticulum compared to the weight of the omasum and abomasum suggest that the development of the rumen was small. The fact that after feeding riboflavin, the animal gained weight, improved in physical appearance and excreted in the urine

only a portion of the riboflavin administered is further evidence that this factor is needed by the calf.

The absence of lesions in the sciatic or brachial nerves, and possibly in the eye, may be due to the short period of time that the animals were on the deficient diet.

SUMMARY

1. The bovine species requires riboflavin, supplied either in the diet or by rumen or intestinal synthesis. On a riboflavin deficient diet the calf develops definite pathological symptoms and riboflavin practically disappears from the urine.

2. Riboflavin deficiency in the young dairy calf is characterized by hyperemia of the buccal mucosa, lesions in the corner of the mouth, along the edges of the lips and around the navel, loss of appetite, poor growth, scours, excessive salivation and lachrymation, and loss of hair. No vascularization of the cornea or opacity of the lens were observed on periodical examination with the ophthalmoscope.

3. The administration of riboflavin rapidly cures the deficiency.

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THE FORMATION OF CAPILLARIES AND OTHER TISSUE CHANGES IN THE CORNEA OF THE METHIONINE-DEFICIENT RAT

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TWELVE FIGURES

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Corneal vascularization is a striking development which may appear with nutritional deficiencies. Hall, Sydenstricker, Hock and Bowles ('46) have described the appearance of corneal vascularization in rats fed a diet lacking protein. A similar vascularization may result from deficiencies of lysine or tryptophane (Totter and Day, '42). In a comprehensive investigation of the changes in morphology resulting from amino acid deficiencies Maun, Cahill and Davis ('45a, '45b) observed that while a diet deficient in phenylalanine resulted in no significant ocular changes, leucine deficiency caused pronounced lesions of the anterior portion of the eye. The corneal epithelium was thinner and there was a loss of cellular polarity. The epithelial cells appeared to be in flattened layers with keratinization on the surface. The basal cells stained more deeply with hematoxylin and showed increased numbers of mitoses. The substantia propria appeared to have lost its fibrillar structure and become homogenous and was thickened. Prominent dilated blood vessels were observed beneath the

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epithelium and in the superficial portion of the substantia propria. Around these and in the adjacent stroma small collections of granulocytes were present. Descemet's membrane was slightly thickened. The vascularization and the epithelial metaplasia were observed to be more evident near the limbus. The same changes were observed in rats on a histidine-deficient diet although the changes were less striking (Maun, Cahill and Davis, '46). The structure of the substantia propria was preserved though it also appeared thickened. The leukocytic infiltration of the perivascular tissue was considerably less.

Glynn, Himsworth and Neuberger ('45) investigated the pathology resulting from deficiency of the sulfur-containing amino acids but made no report concerning the eyes of their animals.

That methionine deficiency in the rat may result in corneal vascularization has already been reported by Sydenstricker, Hall, Hock and Pund ('46). The present study is concerned with a more comprehensive investigation of the corneal changes which occur in this deficiency.

EXPERIMENTAL

Rats of a Wistar strain were placed on the experimental diets when from 21 to 32 days of age. The composition of these diets is given in table 1. Since Hall and Sydenstricker ('47) have pointed out that 9% casein diets such as diets 25 and 36 are suboptimal with respect to histidine, lysine, valine, threonine and tryptophane, groups of rats were placed on diets to which adequate amounts of these amino acids had been added (diets 38 and 39). The animals which received the diet containing 9% of casein (diet 25) were also compared with groups which received diets containing 10 and 11% casein (diets 22 and 23) and the control animals (those receiving diets 36 and 38) were compared with a group which received a 20% casein diet (diet 31). Where possible, littermates were divided between the various diets.

As a further control, dl methionine was fed to 3 rats having corneal vascularization in order to induce a regression of the corneal vascularization.

Also included in table 1 are figures showing the incidence of corneal vascularization in the groups of rats in the various diets as well as the changes in weight which occurred during the first 7 weeks on the diet. It is to be noted, however, that in

TABLE 1

Composition of diets, with the associated incidence of corneal vascularization and rates of growth.[1]

DIET NO.	25	22	23	36	38	39	31
	Gm per 100 gm of diet						
Casein [2]	9.0	10.0	11.0	9.0	9.0	9.0	20.0
dl Methionine				0.5	0.4		
Amino acid mixture [3]					1.57	1.57	
Sucrose	81.8	80.8	79.8	81.3	79.83	80.23	70.8
No. of rats on diet	42	6	10	10	8	9	10
No. showing corneal vascularization	21	5	2	0	0	8	0
Growth, gm per day	0.5	0.2	0.0	0.9	3.1	0.7	3.0

[1] All the above diets contained in each 100 gm: salt mixture 4 gm, cod liver oil 2 gm, cottonseed oil 3 gm, choline chloride 0.2 gm, calcium pantothenate 2.0 mg, pyridoxine hydrochloride 0.4 mg, thiamine hydrochloride 0.4 mg, riboflavin 1.6 mg. The salt mixture was the same as that used by McKibben, Madden, Black and Elvehjem ('39).

[2] Vitamin test casein, General Biochemicals, Inc.

[3] Amino acids added were 0.25% of l (−) histidine hydrochloride, 0.52% of l (−) lysine hydrochloride, 0.30% of dl valine, 0.40% of dl threonine and 0.40% of dl tryptophane.

the groups of rats on the deficient diets, many died during this period. A description of the methods used in caring for the animals, techniques used in biomicroscopic examination of the rats' eyes, and of the method used in making injected corneal preparations is given in an earlier paper (Bowles, Allen, Sydenstricker, Hock and Hall, '46).

For morphologic studies, eyes showing changes typical of methionine deficiency were selected with the use of the bio-

microscope. After the animals were killed, the eyes were fixed in 1:10 formalin for 24 hours, following which the lenses were removed through incisions in the posterior portion of the eyes to facilitate sectioning. No histological changes in the lens had been observed to occur as a result of the methionine deficiency. The tissue next was placed successively in 80%, 95% and absolute ethyl alcohol for periods of 1 hour each, after which it was cleared in Cedar oil for a minimum of 48 hours, or until such time as the procedure was to be completed. After immersion in paraffin for 12 hours at 55°C., the tissues were blocked and serial sections 7 microns thick were cut from a representative area of the cornea. The sections were stained with the Harris hematoxylin stain followed by an eosin counterstain.

Flat preparations also were made from quadrants of corneas of methionine-deficient rats using the method of Buschke, Friedenwald and Fleischmann ('43). One such preparation was made from a cornea the capillaries of which had been injected with India ink in the manner described by Bowles, Allen, Sydenstricker, Hock and Hall ('46). All flat preparations were stained with Harris hematoxylin stain followed by an eosin counterstain.

The histological studies reported here were all made on corneas from methionine-deficient rats which had been fed diet 39, although no differences were observed between the histological changes resulting from consumption of this diet and the other methionine-deficient diets. Four of the rats had been on the diet for about 4 months. The other 5 on this diet died after intervals varying from 5 weeks to 4 months.

The normal variations in the histology of the cornea in this strain of rats had been determined by the study of a number of normal-appearing corneas from control and stock rats.

As in previous studies our use of the term "corneal vascularization" is confined to the situation where there is an actual invasion of the cornea by capillaries. In another paper we have described the normal variation in vascularity

at the limbus in the rat (Bowles, Allen, Sydenstricker, Hock and Hall, '46).

RESULTS AND DISCUSSION

Gross examination of the corneas of the methionine-deficient rats revealed no observable changes other than a transient, diffuse haziness of the cornea. This corresponded to a moderate degree of opacity which was observed with the biomicroscope preceding the invasion of the cornea by capillaries. In general, the capillary invasion and the regression of the corneal vascularization when the rats were changed to the control diet, as observed with the biomicroscope, were similar to what had been described as occurring in protein deprivation (Hall, Sydenstricker, Hock and Bowles, '46). The principal deviation was that the vessels were usually much smaller and often less numerous than the corneal vessels observed in the protein-deficient rats although eventually, in some of the rats, the vessels attained a comparable size. In many rats which had been on the methionine-deficient diet for a month or more, the vessels were very small, and barely visible with the biomicroscope. Frequently they were only partially filled or bloodless. This might be due to a reduction in blood volume similar to that observed by Metcoff, Favour and Stare ('45) and by Benditt, Straube and Humphrey ('46) in rats fed diets very low in protein, or it might possibly be due to spontaneous regression of the vascularization. Glynn, Himsworth and Neuberger ('45) observed hypoproteinemia in their rats deficient in the sulfur-containing amino acids which it is presumed might result in a reduced blood volume. Except for the haziness of the cornea just preceding the formation of the capillaries, the corneas usually appeared to be clear and transparent.

Photophobia was commonly observed in the experimental animals showing corneal changes, as has been noted in other nutritional deficiencies affecting the cornea.

Figures 1 through 4 are pictures of injected rat corneas with different degrees of vascularization. The size of the

vessels seen in figure 5 is characteristic of a large proportion of the corneal vascularization seen in biomicroscopic examination of methionine-deficient rats. The general pattern of vascularization in the corneas of the methionine-deficient rats seemed to be very similar to that observed to result from protein deprivation (Hall, Sydenstricker, Hock and Bowles, '46) though the capillaries were usually much smaller. The peculiar pattern of vascularization shown in the cornea in figure 6 is exceptional. Since this pattern of vascularization was observed in only one eye of one of the methionine-deficient rats, it is quite possible that it may have been due to trauma of some sort, although no evidence of trauma was seen during weekly biomicroscopic examinations or in the injected preparation. Figure 4 is a picture of the cornea of the other eye of this rat.

It will be noted in examining the pictures of the injected corneas that a number of vessels may be seen which are unfilled or are poorly filled with the injection fluid. In most cases these vessels, as well as some which did fill with the injection fluid, were those frequently observed to be bloodless on biomicroscopic examination when the deficiency had reached an advanced stage. The distal ends of many of the capillaries seen in the pictures were probably in many instances, at least for a short distance, newly formed capillaries, as yet nonfunctional. Figure 3 shows not only vessels which were not filled with the injection fluid when the injection was made, but also shows the beading such as was observed during the process of regression of vascularization in protein-deficient rats when these rats were returned to an adequate diet (Hall, Sydenstricker, Hock and Bowles, '46). This rat had been continuously on the deficient diet from the beginning of the experiment until the time when the injection preparation was made. It is possible that this represents a partial spontaneous regression of the vascularization. The 9% casein diets which we used contained 0.22 to 0.30% of methionine as compared to the rat's requirement of 0.6% in the diet (Hall and Sydenstricker, '47).

The plexus of very fine vessels such as may be seen in the middle of the picture in figure 4 is such as is frequently observed in corneal vascularization resulting from amino acid deficiencies.

On examining the histological sections of the cornea, the most constant finding was edema of the epithelium and substantia propria. In the epithelium the edema was both intracellular (fig. 7) and intercellular (fig. 8).

Karyorrhexis, or swelling of the cells with granular disintegration of the nuclear chromatin, was present and almost always in the outer 3 cell layers of the corneal epithelium.

Bowman's membrane was rarely affected and when it was, it was usually slightly displaced in a wavy fashion (fig. 7).

Vascularization was most frequently characterized by subepithelial capillaries (fig. 9) and the more marked the vascularity, the deeper the vessels were found in the stroma. Usually the vessels were seen at approximately the position observed with the biomicroscope. In flat preparations uncanalized vessels were seen and these undoubtedly were the same structures which, when seen with the biomicroscope, were described as fine, white lines (fig. 11).

The vascularization in the cornea which we observed in methionine deficiency was similar to that described by Maun, Cahill and Davis ('46a, '46b) for leucine and histidine deficiencies, though the detailed cellular changes do not coincide. However, it must be pointed out that our study was made with rats which had been for a much longer period on a diet less drastically deficient than those used by Maun, Cahill and Davis.

After methionine-deficient rats had been returned to the control diet and vessels were no longer visible on biomicroscopic examination, small bloodless vessels still could be found in histological sections.

The term "corneal corpuscle" is generally used to refer to the normal cell elements of the substantia propria, of which there are 2 forms: (1) fixed supporting cells, and (2) wandering phagocytes. Our observations indicate that under certain

conditions a third form may arise, this form being by function a primitive endothelial cell, as is indicated by the following observations. On examination of the area of formation of the new capillary, increased numbers of cells were always observed distal to the end of the new capillary. These cells were similar to the normal corneal corpuscle of the phagocytic type, but varied somewhat in size and shape. There were present all gradations from the usual corneal corpuscle to a cell type which was larger and longer. This latter type appeared identical with the endothelial cells seen in the capillary walls. As seen in serial sections, these cells were irregularly arranged and were seen at various depths. However, in flat corneal preparations, the integration of these cells could be followed from an irregular arrangement to their final organization into a functional capillary. This integration may be seen in figures 10, 11 and 12. Figure 10 is from a photomicrograph of a flat preparation of a cornea, the capillaries of which had been injected with India ink. The area shown in the picture includes the branched end of a capillary at the farthest point reached by the injection fluid. As may be observed in this picture, when the side walls of the capillary are in focus, they may be seen as orderly lines of cells. On following the capillary towards the distal end, a point is reached where it becomes bloodless and the lines of cells forming the walls are less orderly in arrangement, as may be seen in figure 11. The 2 lines of cells forming the walls may diverge slightly, and from this point the cells are spaced farther apart and become progressively more irregular in arrangement, until only scattered cells may be observed, as in figure 12. Study of the section from which figure 12 was made showed all gradations in size and shape from typical phagocytic type corneal corpuscles to the formed endothelial cells, as previously described.

That endothelial cells may be derived from corneal corpuscles may be supported on an embryological basis. It is generally accepted that in the embryo amoeboid mesenchymal

cells move in between the optic cup and the epidermis to form, through differentiation, the substantia propria, which further differentiates to form Bowman's and Descemet's membranes. The corneal corpuscles of the substantia propria are apparently these primitive mesenchymal cells, some of which are differentiated to form the supporting cells, and some of which are histiocytes which retain their primitive amoeboid nature. These histiocytes may function as phagocytes, or may further differentiate into endothelial cells when capillaries are needed. In this instance, it is suggested that with methionine deficiency, the nourishment of the avascular corneal tissue becomes inadequate and an attempt is made by the tissue to compensate for the inadequacy by the development of capillaries to bring additional nourishment.

Of the 9 rats from 4 litters on diet 39, 1 died before corneal vascularization appeared. The remaining 8 all developed definite and extensive corneal vascularization in from 6 to 102 days (mean, 35 days). This diet (diet 39), where the nutritional deficiency was more specific, resulted in a rather prompt production of extensive corneal vascularization in 8 out of 9 rats while the other methionine-deficient diets, where the deficiency was less specific, resulted in corneal vascularization in only about half of the rats. The corneal changes resulting with these diets were frequently less extreme and more variable in time of appearance. However, there were no qualitative differences in the corneal changes produced in the various methionine-deficient diets. It is of interest that complete starvation does not produce corneal vascularization (Bessey and Wolbach, '39) although elimination of all protein from an otherwise adequate diet results in prompt and extensive corneal vascularization (Hall, Sydenstricker, Hock and Bowles, '46).

As in rats deprived of protein (Sydenstricker, Hall, Hock and Pund, '46), the repeated administration of large doses of riboflavin had no observable effect on the corneal vascularization resulting from methionine deficiency.

The growth of the rats on the various methionine-deficient diets used seemed to depend more on the age at which the rats were started rather than on the identity of the diet.

None of the control rats showed any significant corneal changes.

SUMMARY

The development of corneal vascularization in rats fed a diet deficient in the sulfur-containing amino acids was followed with the biomicroscope. Histologic studies confirmed these observations. Vascularization was noted first in the subepithelial tissue, and when pronounced, vessels appeared in the deeper portions. Non-canalized capillaries were observed. A difference in the cellular components of the substantia propria was noted and the significance of the endothelial-like cells as to their relation with newly formed capillaries is discussed.

Degenerative changes of the epithelial cells of the cornea are described.

No histologic changes in the lenses were seen.

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PLATE 1

EXPLANATION OF FIGURES

(All 15 × reduced approximately one-third)

1 through 5 Oblique views of corneas of methionine-deficient rats, which have been injected with India ink. These rats had been on a methionine-deficient diet for 116, 116, 200, 40 and 160 days, respectively, when the injection preparation was made, although corneal vascularization had been observed with the biomicroscope in these rats after 39, 99, 180, 29 and 98 days on the deficient diets.

6 Anterior view of the injected cornea from the other eye of the rat, the cornea from which is shown in figure 4.

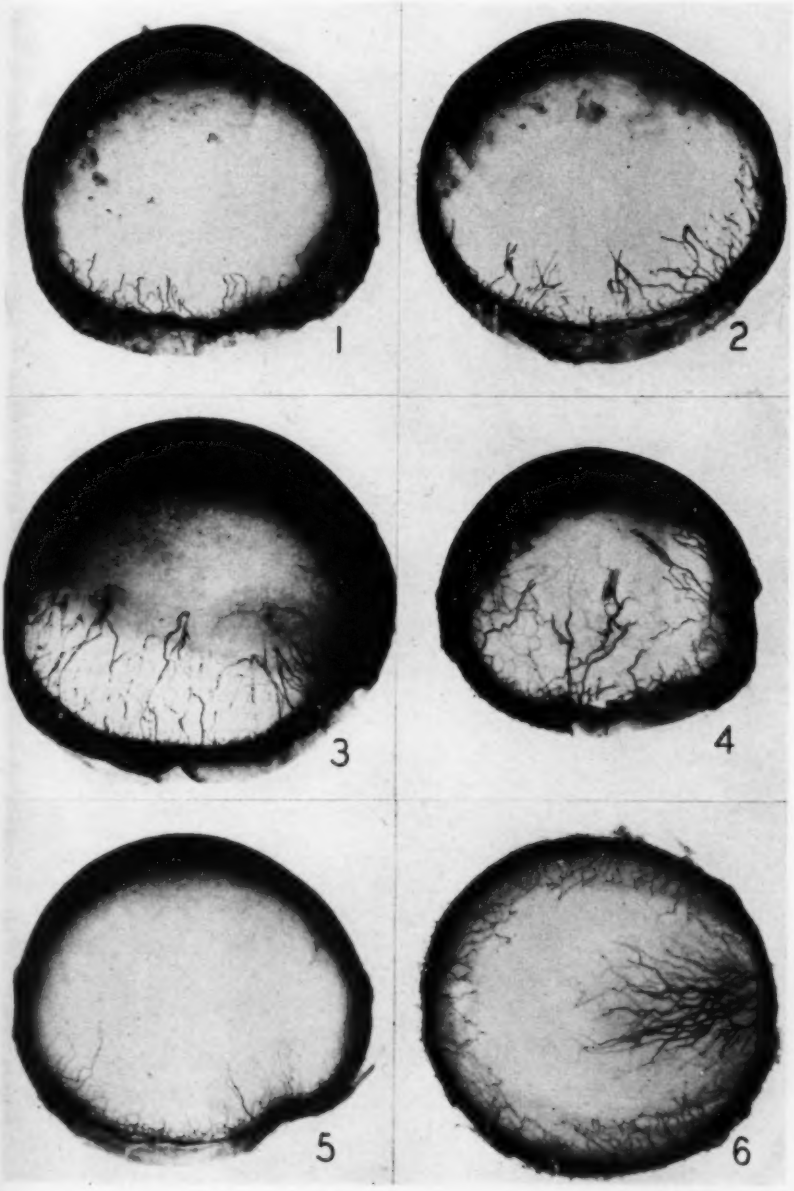
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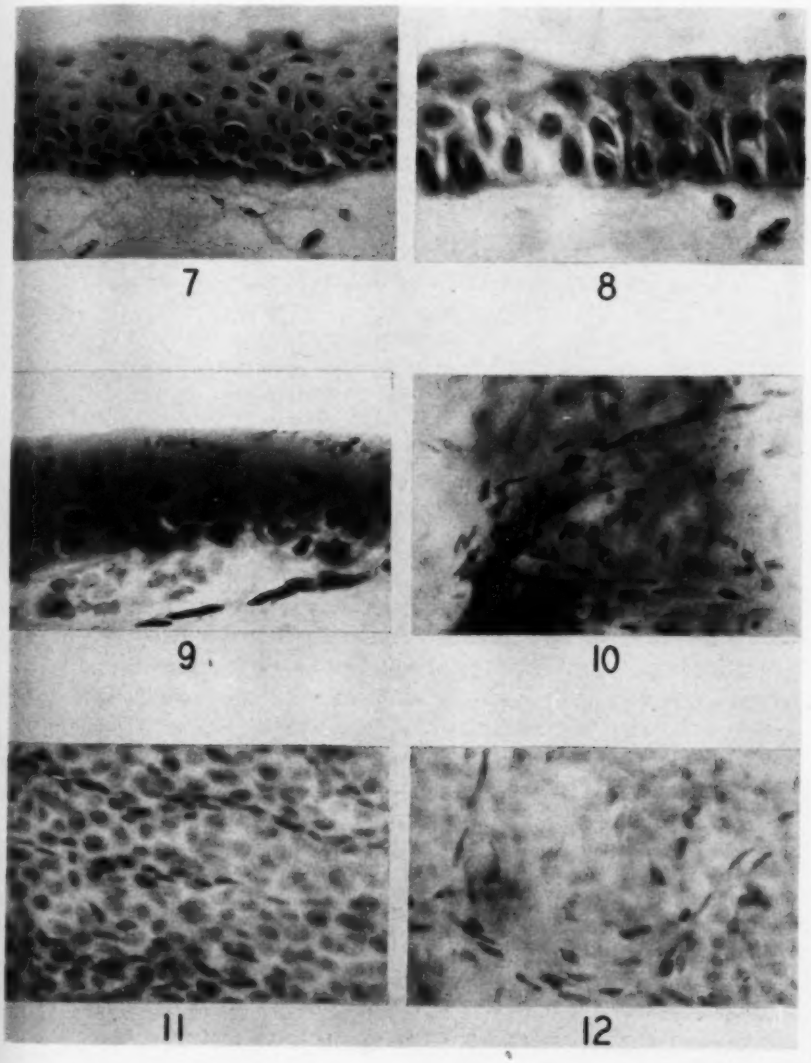
PLATE 2

EXPLANATION OF FIGURES

7 through 9 Sections of corneas of methionine-deficient rats. Figure 7 shows intracellular edema of the epithelial cells and wavy displacement of Bowman's membrane. Figure 8 shows early intercellular edema of the epithelial cells. Figure 9 shows focal areas of karyorrhexis of the nuclear chromatin of the outer layers of cells, pyknosis of the nuclei of the inner layers of cells, and a large subepithelial capillary. (Figure 7, 600 ×; figs. 8 and 9, 1400 ×. All reduced approximately one-third).

10, 11 and 12 Flat preparations of corneas showing capillary formation. (Figure 10, 430 ×; figs. 11 and 12, 700 ×. All reduced approximately one-third).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WORK PERFORMANCE, RESPIRATORY EXCHANGE, AND CERTAIN BLOOD CONSTITUENTS AFTER ISOCALORIC MEALS OF LOW AND HIGH CARBOHYDRATE CONTENT [1,2]

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ONE FIGURE

(Received for publication October 19, 1946)

In previous studies (Haldi and Wynn, '46a, b) it was found that work performance in severe exercise of short duration was not affected by either the amount or kind of food ingested several hours before exercise. Hypoglycemia with a concomitant diminution in the capacity for work, believed by some to be the resultant of a high carbohydrate intake (Wilder, '43; Thorne, Quinby and Clinton, '43) was not observed in any of our subjects after a meal rich in carbohydrate. The limiting factor of endurance for brief exhausting work, however, may not be the same as for heavy work that can be carried on for a longer time before exhaustion. This consideration suggested the advisability of the present study on work performance after different types of meals with the work load so adjusted that exercise could be continued for a much longer time than in our previous experiments.

The subjects were 3 adult males, 2 adolescent males, and 1 adult female, ranging in age from 14 to 37 years and in weight from 120 to 200 pounds. This purposeful diversification in the

[1] Preliminary report: Fed. Proc., '46; *5:* 39.

[2] Acknowledgment is made to the Sugar Research Foundation for a grant-in-aid for conducting this investigation.

choice of subjects was designed to determine whether there were any differences in response to the 2 types of meals that might be related to age or sex. All the subjects were in splendid physical condition and accustomed to 1 form or another of athletic activity.

PROCEDURE

For a period of about 3 weeks each subject was thoroughly trained in riding the Prony-brake bicycle ergometer against a load of such magnitude that he was able to continue the exercise from 12 to 18 minutes before becoming completely exhausted. This load, which had been determined for each subject by a number of trials, was maintained constant throughout the experiments. In the training program the subjects while riding the bicycle were connected with the metabolism apparatus through the mouthpiece and nose clip. The apparatus used in these experiments has been described elsewhere (Bachmann and Haldi, '37). At the conclusion of the training period the amount of work done was fairly constant from day to day. Blank experiments were then run to familiarize the subject with the entire procedure.

In the earliest stages of this study we were concerned over establishing valid criteria of complete exhaustion. It was soon found, however, that with the whole-hearted cooperation given by the subjects this matter presented no serious difficulties. Each subject continued riding until he reached a point where it was impossible with maximum effort to push down the pedal on the bicycle. The speed of riding as shown by a speedometer remained constant throughout the experiment. As the rider neared exhaustion there was observed a profuse perspiration over the entire body and twitching of the muscles. On a number of occasions the subjects experienced severe cramps in the calves of the legs.

On the day of the experiment the meal prepared in the University cafeteria by competent dietitians was served at noon. Each meal supplied approximately 1050 cal. Carbohydrate, protein, and fat provided, respectively, 76, 13, and 11%

of the total calories in the high carbohydrate meal and 13, 37, and 50%, respectively, in the low carbohydrate meal. All the experiments on 1 subject were completed before beginning with another. Two experiments were done each week, 1 on each of 2 different types of meals, and were so spaced as to leave several days intervening. On these intervening days the subject rode the bicycle ergometer against the customary load for 10 minutes in order to keep himself in training. There were 6 experiments on each subject with each kind of meal, making a total of 72 experiments.

One and a half to 2 hours after the meal the subject reported to the laboratory and reclined on the metabolism couch for 30 minutes. He was then connected to the metabolism apparatus and the respiratory exchange determined for 2 10-minute periods. At the conclusion of these base-line periods blood samples were drawn by finger puncture for sugar determinations on all the subjects and also for lactic acid analyses on 4 of them. The subject then mounted the bicycle ergometer, adjusted the mouthpiece so as to expire into the spirometer and at a given signal started pedalling at the rate of 56 revolutions per minute. He continued at this rate until exhausted, whereupon he rolled off the bicycle onto the adjacent couch without being disconnected from the metabolism circuit. Readings on the counter of the ergometer were taken after 10 minutes riding and again at the conclusion of the exercise. The subject rested for 10 minutes in the recumbent position and then mounted the bicycle for the second time and rode again until exhausted. At the end of this second exercise period he reclined on the couch while determination of the respiratory exchange was continued for 3 10-minute recovery periods. Blood samples were drawn immediately after the 2 exercise periods and again at the end of 30 minutes of recovery. Analysis for blood sugar was made by the Hagedorn-Jensen procedure (Peters and Van Slyke, '32) and for lactic acid by the Barker-Summerson method ('41).

RESULTS

Work output

The amount of work done varied from 1 subject to another but within a relatively narrow range for 4 of them: W.W., C.D., J. L., and J.S. The 1 female subject accomplished only about 65% as much work as these 4 whereas 1 adolescent male did 20% more work than the other 4 males. These latter results are of interest for here we have a youth 14 years old with a much greater capacity for work than was manifested

TABLE 1

Work output and per cent recovery.[1]

Subject	HIGH CARBOHYDRATE MEAL			LOW CARBOHYDRATE MEAL		
	Work done		Recovery	Work done		Recovery
	First period	Second period		First period	Second period	
	kg m	*kg m*	%	*kg m*	*kg m*	%
W.W.	13,993	6,933	49.5	13,456	6,856	51.0
J.L.	14,435	8,076	56.0	13,666	6,713	48.9
C.D.	13,643	7,096	52.0	13,324	7,802	58.6
J.H.	16,819	9,590	56.9	16,374	9,311	56.9
J.S.	13,830	7,054	50.9	13,397	6,583	48.9
E.H.	9,128	4,892	53.6	9,082	5,334	57.6
Average	13,641	7,273	53.2	13,216	7,100	53.7

[1] Each figure in the table is an average of 6 experiments except the final averages which are derived from 36 experiments.

by a robust adult (J.L.) who had engaged in professional football only a short time previous. The other adolescent (J.S.) who weighed only 130 lbs. and the adult (C.D.) weighing 120 lbs. were able to do almost as much work as the heavier football player. Obviously muscle mass was not the sole factor determining work output.

Inspection of the data in table 1 shows a uniformity of results with all the subjects on the 2 types of meals. The average amount of work done in the first exercise period and in the first and second periods combined was approximately 3% more after the high than after the low carbohydrate meal.

This difference, however, was not statistically significant. There was likewise no significant difference in the percentage recovery after the 2 meals. The percentage recovery was determined by multiplying the work done in the second period by 100 and dividing the product by the work done in the first period.

The double work period was the method of choice not only because it provides more information than the single work period, but also because it is held by some investigators (Foltz, Ivy and Barborka, '42) that the percentage recovery is less variable in untrained individuals and consequently more advantageous for the study of fatigue than a single work period. With our subjects, however, it was found that the average of the coefficients of variation for the work done during the first exercise period was 6.0 as compared with a coefficient of 13.7 for the percentage recovery. By this criterion the work done in the single period was 44% as variable as the percentage recovery.

The differences in our results as compared with those of Foltz and others ('42) were not due to an unusually high coefficient in the percentage recovery which was 13.7 in our subjects as compared with their coefficient of 11.4 but to our low coefficient of 6.0 in the work output of the first period as against their coefficient of 13.2. While the double work period has the advantage of supplying more information than the single work period, our results indicate that generalization on the relative value of the two methods in the study of fatigue should be withheld pending further investigation. It may be that the explanation of the difference between our results and those of Foltz and his associates lies in the fact that our subjects were all of the athletic type and customarily took part in some form of athletic activity. Apparently they reached the peak of training in a very short time. Consideration should also be given to the possibility that the factors responsible for recovery from fatigue may not be identical with those that determine the onset of fatigue.

Muscular efficiency

The net muscular efficiency was calculated by dividing the heat equivalent of the work by the difference in the amount of heat produced during exercise and the amount produced during the same length of time in the resting state before exercise. Heat production was determined from the respiratory exchange. As shown in table 2, the net muscular efficiency was the same after both types of meals. These results are in

TABLE 2

Net muscular efficiency.[1]

SUBJECT	HIGH CARBOHYDRATE MEAL PERIODS			LOW CARBOHYDRATE MEAL PERIODS		
	First exercise		Second exercise	First exercise		Second exercise
	First 10 min.	Remainder		First 10 min.	Remainder	
	%	%	%	%	%	%
W.W.	25.7	22.7	25.4	24.6	22.6	25.7
J.L.	23.2	19.2	21.9	22.2	19.6	22.2
C.D.	22.3	20.3	22.5	22.3	20.2	22.6
J.H.	21.3	20.0	20.8	21.1	19.7	20.8
J.S.	23.9	22.9	23.6	23.8	21.3	23.3
E.H.	23.2	21.6	23.6	21.0	20.5	21.5
Average	23.3	21.1	23.0	22.5	20.7	22.7

[1] Each figure in the table is an average of 6 experiments except the final averages which are derived from 36 experiments.

agreement with those that have been reported previously (Haldi, Bachmann, Ensor and Wynn, '38). During the latter part of the first exercise period in the experiments with both the high and the low carbohydrate meals it was approximately 2% lower than in the first 10 minutes of exercise. This difference between muscular efficiency in the earlier and later stages of the first exercise period was statistically significant from which it may be concluded that fatigue is associated with a lowering of muscular efficiency. In the second period, after a short rest, the muscular efficiency was the same as in the first 10 minutes of exercise.

Blood sugar

In the resting state immediately before exercise the blood sugar level was appreciably higher after the high carbohydrate than after the low carbohydrate meal (table 3). At the conclusion of both the first and second exercise periods the blood sugar had dropped from 145 to 106 mg % in the experiments on the meal rich in carbohydrate, whereas it remained at practically the resting level in those with the low carbohydrate meal. At the end of the recovery periods the blood sugar concentration was the same in both sets of experiments.

TABLE 3

Blood sugar concentration.[1]

	HIGH CARBOHYDRATE MEAL				LOW CARBOHYDRATE MEAL			
Subject	Resting	End of first exercise period	End of second exercise period	End of recovery period	Resting	End of first exercise period	End of second exercise period	End of recovery period
	mg %	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*
W.W.	128	91	97	121	114	103	100	118
J.L.	166	116	120	119	128	137	135	125
C.D.	132	100	104	113	119	119	112	119
J.H.	130	107	108	126	123	112	112	118
J.S.	168	112	111	148	152	130	126	134
E.H.	138	112	102	109	116	119	118	113
Average	145	106	107	123	125	120	118	122

[1] Each figure in the table is an average of 6 experiments except the final averages which are derived from 36 experiments.

These data may be taken to indicate that more energy was supplied by glucose after the large than after the smaller intake of carbohydrate. In the experiments with the high carbohydrate intake the rate of glycogenolysis during exercise apparently did not keep pace with the rate of glucose utilization resulting in a drop in blood sugar from 145 to 106 mg %. Glycogenolysis during recovery appears to have proceeded faster than glucose utilization thereby raising the blood sugar level to 123 mg %. After the meal of a smaller carbo-

hydrate content glycogenolysis and glucose utilization were apparently in equilibrium.

Blood lactic acid

The lactic acid concentrations of the blood drawn at the end of the rest period immediately before exercise were 15.9 ± 5.1 and 10.0 ± 3.6 mg %, respectively, in the experiments on the high and low carbohydrate meals. The difference of 5.9 mg % must be regarded as statistically significant since it was 4 times the critical ratio. Immediately after the first and second exercise periods the blood lactic acid level was appreciably above the base line and practically the same in the 2 sets of experiments namely, 48.0 and 44.8 as compared with 46.5 and 41.4 mg %. At the conclusion of the experiment it had returned to 4 and 6 mg %, respectively, above the base level in the 2 groups of experiments.

The initial higher percentage of blood lactic acid after the meal rich in carbohydrate is believed to be due to the availability of a greater amount of glucose and fructose to the organism than after the low carbohydrate meal. It has been shown that the ingestion of both glucose and fructose lead to an increase in blood lactic acid concentration (Bachmann and Haldi, '37; Edwards, Bensley, Dill and Carpenter, '44).

Respiratory quotient

After the low carbohydrate meal the average respiratory quotient in the resting state was 0.79 (fig. 1). During the first 10 minutes of the first exercise period it rose to 0.97 and went slightly higher during the last few minutes of exercise. In the second exercise period the quotient was somewhat lower, at 0.91. These results are taken to indicate that the major portion of the energy of exercise was derived from carbohydrate but to a lesser degree in the second than in the first period. This may reasonably be assumed to be due to a reduction of the carbohydrate reserves in the first exercise period.

After the meal rich in carbohydrate the base line of the respiratory quotient was considerably higher than after the low carbohydrate meal. During the exercise and recovery periods the quotient was also at a higher level than in the experiments with the low carbohydrate meal. In the first exercise period it rose slightly above unity. Quotients of this magnitude during exercise have been reported by other investigators (Carpenter and Fox, '31). In the second 10-minute

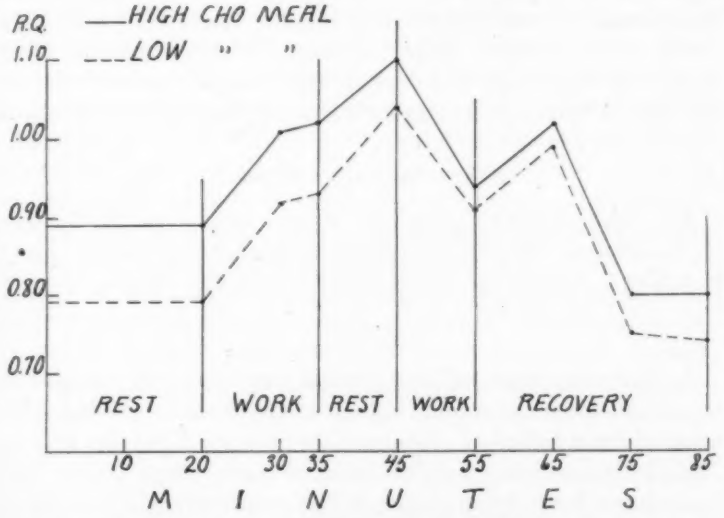

Fig. 1 The respiratory quotient during rest, exercise, and recovery after a high and a low carbohydrate meal. The 2 exercise periods in the graph are approximate averages of the length of time the subjects were able to ride the bicycle until exhausted.

period after the final exercise period the quotient descended rapidly to a level slightly below the base line and remained at this level during the last 10-minute period of the experiment. A similar drop in the quotient occurred in the experiments with a low carbohydrate meal.

From these observations and those on blood sugar it may be concluded that as a result of a larger intake of carbo-

hydrate there was more energy supplied by this food material both at rest and during exercise.

In both sets of experiments there was a marked rise in the respiratory quotient immediately after exercise which in some instances exceeded unity. This phenomenon has been observed in previous experiments of a similar nature in our laboratories (Haldi and Bachmann, '37). Analysis of the data of the former experiments led the authors to suggest that the high quotients might have been due to an acceleration of the transformation of glucose into fat by exercise which persisted a few minutes after exercise. This explanation, however, we wish to regard as only tentative until various etiological possibilities can be explored by further experimentation.

General observations

The relative work output, percentage recovery, blood sugar and muscular efficiency, after the 2 types of meals, as shown in tables 1–3, were the same regardless of age or sex.

DISCUSSION

In the treatment of the clinical syndrome of functional hypoglycemia a reduction in the carbohydrate intake has been found in a number of instances to give good results (Conn, '40). It is probably on the basis of such observations that some have been led to believe that relatively high carbohydrate meals predispose normal individuals to hypoglycemia with a concomitant feeling of weakness and a diminution in the capacity for work. The present experiments on normal healthy adolescents and adults do not support this point of view. Several hours after the ingestion of a high carbohydrate meal there was no evidence of hypoglycemia either before or immediately after severe exhausting exercise. Work output, percentage recovery, and muscular efficiency were not diminished by the relatively large carbohydrate intake. These observations afford confirmatory evidence for the conclusion drawn from previous studies (Haldi and Wynn, '46a) that

the hypoglycemic syndrome of functional origin is a pathological condition and not a normal physiological response to an antecedent high carbohydrate intake.

We are unable, at present, to account for the apparent discrepancy between our observations and those of D'Angelo ('46) who found that the ingestion of 70 or 150 gm of dextrose induced the development of "hypoglycemic" reactions in 5 out of 6 subjects approximately 50% of the total number of times tested. These reactions usually occurred 3 to 5 hours after ingestion of the sugar with approximately equal frequency at either ground level or altitude. "The seizures were characterized by subjective sensations of nervousness, impending danger, paresthesia, weakness and hunger, and objective manifestations of pallor, sweating, tremulousness, coldness of the extremities and a tendency in some toward a drop in oral temperature." It is of interest to note, however, that in most instances the blood sugar level was within the normal range at the time of the so-called hypoglycemic reaction.

None of our subjects manifested any of these symptoms. They all reported that they could observe no difference on the 2 types of meals in their subjective state or in their general physical condition at any time during the experiments. At the conclusion of each experiment they experienced a sense of weakness in the legs and knees but this soon wore off so that they were able to proceed in their usual activities without discomfort. One of them (J.H.) invariably went from the laboratory to the gymnasium where he engaged in an hour's practice in basket ball.

CONCLUSIONS

Work output, muscular efficiency, and percentage recovery (measured by a comparison of the work performance in the first and second exercise periods) were not diminished by a high carbohydrate intake 2 to 3 hours before exercise.

After the meal rich in carbohydrate the blood sugar level immediately before exercise was higher than at the corresponding time after the low carbohydrate meal.

The data on the blood sugar levels and the respiratory quotient are interpreted as indicating that a greater portion of the energy of exercise was derived from carbohydrate in the experiments with a high carbohydrate meal than after the low carbohydrate intake.

There were no age or sex differences in the comparative work performance on the 2 types of meals.

At no time throughout the experiments was there any evidence of the so-called hypoglycemic reaction to the high carbohydrate meal.

The data of these experiments are offered as confirmatory evidence for the conclusion drawn from other observations that a hypoglycemic reaction is not the usual physiological response to a high carbohydrate meal.

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IMPROVING THE NUTRITIVE VALUE OF FLOUR

I. THE EFFECT OF SUPPLEMENTING ENRICHED FLOUR WITH OTHER B-COMPLEX VITAMINS [1]

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FOUR FIGURES

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There is a great demand for cereal products by much of the world's population. A large proportion of the food calories of many people is contributed by bread and cereals. Stiebling, Monroe, Coons, Phipard and Clark ('41) found that grain products furnished 25% of the total calories in the diets of farm families in the northern and western parts of the United States and 38% in the southeastern part. Data collected from government publications by Cummings ('40) showed that bread, flour and cereals provided 19% of the calories in the diets of families of professional men, 27% in well-to-do farm families and 54% in poor farm families in the southern states. Holman ('46) summarized the situation by stating that the proportion of cereals, in diets commonly used, varies from less than 30% of the total in the British and North American diets to 80–90% in the oriental diets.

In recent years, investigations regarding flour and bread have been stimulated by the demand for improvement in the nutritive value of these products. Bread and cereals are important sources of carbohydrates and protein. The proper

[1] Contribution no. 134 of the Department of Home Economics, Kansas Agricultural Experiment Station, Manhattan.

oxidation of the carbohydrates in animal metabolism is dependent upon the presence of a definite quantity of the B-complex vitamins in the diet. Because of this fact it was considered advisable to continue the work on improving the nutritive value of flour by the addition of B-complex vitamins. The literature was reviewed in a previous paper by Westerman and Bayfield ('45). The present paper presents further information regarding this problem. The agents which have been chosen for study are calcium pantothenate, pyridoxine, choline, and the addition of greater quantities of riboflavin and thiamine. At a time when good food sources of the B-complex vitamins such as meat and milk are apt to be low or lacking in the diets of many people, it seems logical that the addition of synthetic vitamins to flour may be a method of taking care of this deficiency.

EXPERIMENTAL PROCEDURE AND RESULTS

The experimental procedure followed that described in the paper by Westerman and Bayfield ('45). Young albino rats weighing between 40–50 gm were used in the 4 experiments reported here. Table 1 gives the detailed composition of the diets.

First experiment: flour and wheat as 50% of the diet

In the first experiment the flour and wheat were fed at a level of 50% in the diet and replaced an equivalent amount of sucrose. The 50% level was chosen because before the war people in many European countries and certain low income groups in the United States consumed diets containing that much or more of cereal foods. The flour was milled from the same lot of wheat that was used in the test. Thiamine, riboflavin and calcium pantothenate assays on the wheat showed 4.1, 1.1 and 7.7 μg per gm, respectively. The pyridoxine content was not determined but Swaminathan ('40) reported 7 μg pyridoxine per gm in wheat and Tepley, Strong and Elvehjem ('42) reported a 50% loss in the milling process.

The rats ate on the average 6 gm of food daily. Since the diet was 50% wheat or flour they consumed approximately 3 gm of these each day. Therefore, the wheat in the diet supplied 12.3 μg of thiamine, 3.2 μg of riboflavin, 23.1 μg calcium pantothenate, and perhaps 21 μg of pyridoxine daily. The flour assayed 4.7, 3.1 and 2.6 μg for thiamine, riboflavin and calcium pantothenate, respectively, and supplied the animals with 14.1 μg thiamine, 9.4 μg riboflavin, 7.7 μg calcium pantothenate and perhaps 10.5 μg pyridoxine daily. Diets IV and V had 10.3 μg of calcium pantothenate added per gm of flour thereby bringing the total daily intake of calcium pantothenate up to 38.6 μg. Diet V had 3.5 μg of pyridoxine per gm added to the flour making the probable daily intake of pyridoxine 21 μg.

After the animals were placed on the test materials, they were weighed every 6 days. The growth test was conducted over a period of 72 days. The average weight gains of each group are shown in figure 1.

Those animals on the B-complex free diet lost weight rapidly and died in about 18 days. Those on ground whole wheat gained on the average 27 gm during the 72-day test, while those on enriched flour gained an average of 30 gm. The animals on enriched flour to which calcium pantothenate was added made an average gain of 44 gm during the test while those with both calcium pantothenate and pyridoxine added showed an average gain of 101 gm. The animals on the stock diet made an average gain of 190 gm during the test.

When 10.3 μg calcium pantothenate per gm were added to the enriched flour, the increase in growth was above that of the groups on enriched flour or whole wheat. This seems to indicate that more calcium pantothenate was needed for growth than that furnished by the enriched flour or the whole wheat. When both 10.3 μg calcium pantothenate and 3.5 μg pyridoxine per gm were added, much better growth was noted, apparently showing a need for both of these vitamins. However, with the vitamins added at the levels indicated, growth was not as good as that of the animals on the stock diet. It

TABLE 1

Composition of the diets.

The basal vitamin B-complex free diet consisted of 20% vitamin free casein, 60% sucrose, 12% fat, 5% salt mixture and 3% cod liver oil. In experiment I the flour and wheat were fed at a 50% level in the diet and replaced an equivalent amount of sucrose. In experiments II, III, and IV 40% of the diet was enriched flour with the B vitamins added to the flour as indicated below.

DIET NO.	EXPERIMENT I Changes in diet	EXPERIMENT II Changes in diet	EXPERIMENT III Changes in diet	EXPERIMENT IV Changes in diet
I	Basal diet	40% enriched flour	40% enriched flour	40% enriched flour
II	50% enriched flour	Added pyridoxine (3.5 μg/gm)	Added pyridoxine (3.5 μg/gm)	Added riboflavin (1 μg/gm)
III	50% ground whole wheat	Added pyridoxine (7 μg/gm)	Added pyridoxine (7 μg/gm)	Added riboflavin (1 μg/gm); and thiamine (1 μg/gm)
IV	Enriched flour + Ca pantothenate (10.3 μg/gm)	Added Ca pantothenate (10.3 μg/gm)	Added Ca pantothenate (10.3 μg/gm)	Added pyridoxine (14 μg/gm)
V	Added Ca pantothenate (10.3 μg/gm); and pyridoxine (3.5 μg/gm)	Added Ca pantothenate (20.5 μg/gm)	Added Ca pantothenate (20.5 μg/gm)	Added choline (3 mg/gm); and pyridoxine (7 μg/gm)
VI	Stock diet	Added pyridoxine (3.5 μg/gm); and Ca pantothenate (10.3 μg/gm)	Added pyridoxine (3.5 μg/gm); and Ca pantothenate (10.3 μg/gm)	Added pyridoxine (14 μg/gm); and Ca pantothenate (40 μg/gm)
VII		Added pyridoxine (7 μg/gm); and Ca pantothenate (20.5 μg/gm)	Added pyridoxine (7 μg/gm); and Ca pantothenate (20.5 μg/gm)	Added Ca pantothenate (40 μg/gm)
VIII		Stock diet		Added choline (3 mg/gm); and Ca pantothenate (20 μg/gm)
IX				Added pyridoxine (14 μg/gm); Ca pantothenate (40 μg/gm); and choline (3 mg/gm)

appears that other factors are involved, perhaps a need for greater quantities of the B-complex vitamins already added or other members of the B-complex group.

At the end of 72 days, the males and the females on the same diet were placed together for breeding purposes. The animals on the stock diet produced normal litters. Of those on the experimental diets, only the animals with calcium pantothenate and pyridoxine added to the flour produced any young. Only half of the females on this diet had young, and these died before maturity.

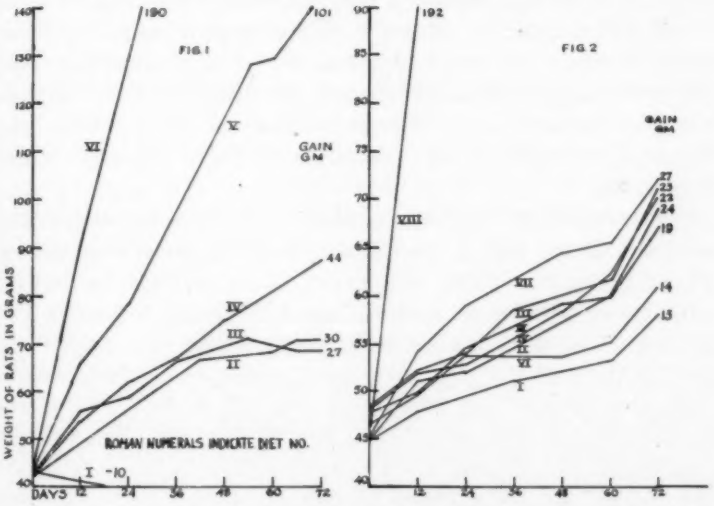

Fig. 1 Growth curves of animals in Experiment I.
Fig. 2 Growth curves of animals in Experiment II.

Second experiment: effect of increasing calcium pantothenate and pyridoxine, with flour as 40% of the diet

In an attempt to obtain better growth and reproduction in the animals, a second experiment was conducted to determine the effect of increasing the quantities of calcium pantothenate and pyridoxine. The amount of flour used in the diet

was 40%. This change was made because it was thought that the American people might consume cereals at a 40% level in the diet but it was doubtful that the 50% level would be reached by a large percentage of the population. The flour was a good grade of enriched flour purchased from the grocer. Analyses showed that it contained 5.8 μg per gm of thiamine, 2.6 μg per gm of riboflavin and 2.0 μg per gm of calcium pantothenate.

There were 8 groups of animals. The composition of the diets is given in table 1. The test covered a 72-day period with the results as shown in figure 2.

On the average, the animals did not gain as much as those in the previous test with the same enriching materials. This may partially be explained by the change from 50 to 40% in the quantity of flour in the diet. Since the flour carried the B-complex vitamins, the quantities of these were therefore decreased.

The growth curves (fig. 2) show that the animals on the enriched flour, diet I, made the least gains. They gained 13 gm during the 72-day test period. Those animals on diet VI with 3.5 μg pyridoxine and 10.3 μg calcium pantothenate per gm added to the enriched flour grew rather rapidly for the first 24 days of the test, then barely maintained their weight until the sixtieth day when they started to gain again, but their total gain was only 14 gm during the test. The animals on diets II, III, IV and V made approximately the same gains throughout the test. Those on diet II, with 3.5 μg additional pyridoxine per gm of flour, showed greater gains during the last 12 days on the test, but as 4 of these animals died, it may be that the stronger ones survived, thus accounting for the greater gain. The rats on diet VII, with 7 μg pyridoxine and 20.5 μg calcium pantothenate added per gm of flour, showed better average gains throughout the test, with a total average gain of 27 gm.

Increasing the amount of pyridoxine alone did not increase the growth rate. The animals on 3.5 μg pyridoxine made an average gain of 24 gm, while those on 7 μg pyridoxine per gm

made an average gain of 23 gm. Increasing the calcium pantothenate did not increase growth. Animals on 10.3 μg per gm gained on the average 22 gm and those on 20.5 μg per gm gained 19 gm. When both the pyridoxine and calcium pantothenate were increased, there was an increase in weight of 13 gm over the animals receiving 3.5 μg pyridoxine and 10.3 μg calcium pantothenate per gm of flour.

The animals on this test were mated for breeding purposes, but no young were born. Then the females were placed with normal males but kept on the same experimental diets. Those on enriched flour alone did not reproduce, those with 3.5 μg pyridoxine per gm added to the flour produced litters, but all the young died or were eaten by the mother within 2 days after birth. The females receiving the 7 μg pyridoxine per gm of flour did not reproduce; this observation was unexpected and is unexplainable. The females on the diet with flour plus calcium pantothenate alone did not produce young while those with flour plus both pyridoxine and calcium pantothenate produced young; however, all the young died within 7 days.

Third experiment: repetition of second experiment with longer feeding period

In the third experiment (fig. 3), 8 animals were used in each series and the diets contained 40% enriched flour in which the added vitamins were incorporated. This test was a partial repetition of the second experiment, but was continued for 90 days. On the average, the animals on this test made better gains than in the previous test. The greatest gains (average 73 gm) were made by the animals on diet VII, which had 7 μg pyridoxine and 20.5 μg calcium pantothenate per gm added to the flour. These were followed by the animals on diet VI, with 3.5 μg pyridoxine and 10.3 μg calcium pantothenate per gm, and also those on diet III, with 7 μg of added pyridoxine with average gains of 56 and 57 gm, respectively. However the curves show that the animals on diet III always gained less than those on diet VI. There was only a slight difference

in the average weight gain of 47 gm by the group on diet V with 20.5 μg calcium pantothenate and the 45 gm average weight gain by those animals on diet II with 3.5 μg pyridoxine, while those on diet IV with 10.3 μg calcium pantothenate per gm made an average weight gain of 42 gm. The animals on enriched flour alone made the least average gain, 38 gm, during the test.

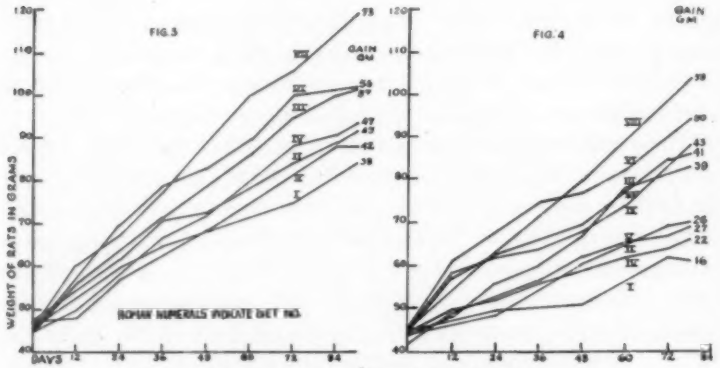

Fig. 3 Growth curves of rats in Experiment III.
Fig. 4 Growth curves of rats in Experiment IV.

Over the 90-day period, it seems that increasing both the pyridoxine and calcium pantothenate had a beneficial result. Increasing the calcium pantothenate alone had some beneficial results while increasing the pyridoxine alone seemed to exert a greater stimulus on growth than did calcium pantothenate.

Fourth experiment: effect of increasing riboflavin and thiamine as well as calcium pantothenate, pyridoxine, and of adding choline

In the fourth experiment, it was decided to test the effect of increasing the amounts of riboflavin and thiamine in the flour each by 1 μg per gm of flour, and also to test both the effect of adding much larger amounts of calcium pantothenate

and pyridoxine and that of adding choline. Table 1 shows the composition of the diets. The test covered a period of 78 days.

The animals on enriched flour alone made the least gain. Their average weight gain during the test was 16 gm. The addition of riboflavin increased the gain to 26 gm, while the addition of both riboflavin and thiamine caused a gain of 39 gm. This was a slightly greater gain than was made by the animals with pyridoxine added or pyridoxine and choline, which made gains of 22 and 27 gm, respectively. The animals with pyridoxine, calcium pantothenate, and choline made approximately the same gain, 43 gm, as those on 40 μg calcium pantothenate per gm which gained 41 gm. The rats receiving 14 μg pyridoxine and 40 μg calcium pantothenate per gm gained 50 gm. This was not as much of a gain as was exhibited by the rats that received choline and 20 μg calcium pantothenate per gm of flour, which made an average gain of 58 gm. The effect of the addition of choline to the enriched flour appeared to be slight, but this might have been expected since the diet contained 20% casein. In all probability the methionine in the casein provided sufficient methyl groups for the formation of choline.

DISCUSSION

Flour enriched at the levels recommended by the National Research Council with thiamine, riboflavin and nicotinic acid, when used as the sole source of the B-complex vitamins does not supply enough of these vitamins to support normal growth in rats when fed at a level of 40 or 50% in the diet. While flour and bread are not necessarily expected to supply all the B-complex vitamins in the diet, it might be well to have more of these vitamins supplied by the cereal foods inasmuch as cereals make up a major portion of the food intake of so many people in the world. There is the added factor of a lack of adequate supply of many of the foods which carry the B-complex vitamins, particularly meat and milk. When milk is available it is used in the making of bread, thereby adding not only thiamine, riboflavin and nicotinic acid but other B vita-

mins. At present it may be questioned whether there is enough milk available for this purpose in the United States; it is certainly not in the rest of the world.

In a report by Milan and Bell ('46) regarding the nutrients in the diets of a North Carolina village population it is stated that pork and milk contributed, respectively, 14.3% and 11.4% of the thiamine in the adult diets while enriched bread contributed 16.7% of the thiamine and dried peas and beans furnished 19.6%. As sources of riboflavin in these diets milk ranked first, contributing 42.2% and eggs second with 11.2%. Enriched bread contributed only 5.2% of the riboflavin in the total diet.

There is the possibility that enriched bread might contribute more riboflavin to the diet. In our experiments it was found that the addition of even a small quantity of riboflavin to the enriched flour increased the rate of growth while the addition of both riboflavin and thiamine gave a still greater growth increase. In all the experiments the addition of pyridoxine or calcium pantothenate alone increased the growth rate but when they were added together still better growth was obtained.

Supplee, Bender and Kahlenberg ('42) found that 20 μg of calcium pantothenate per day were adequate for normal rat growth. In our experiments the animals ate each day, on the average, 6 gm of food which contained 2.4 gm of flour. The flour with 10.3 μg added calcium pantothenate per gm increased the calcium pantothenate in the diet by 24.7 μg. According to Supplee et al. ('42), this was an ample amount of the vitamin. Our data appear to confirm this since increasing the calcium pantothenate in the flour above 10.3 μg per gm did not result in a corresponding weight increase (figs. 2 and 3).

The addition of pyridoxine and calcium pantothenate to enriched flour under the conditions of these experiments appears to have a beneficial effect on the growth rate of the rat. Increasing the added pyridoxine from 3.5 μg per gm to 7 μg per gm in the flour produced increased growth (fig. 3). When both calcium pantothenate and pyridoxine were in-

creased from 10.3 μg per gm to 20.5 μg per gm and from 3.5 μg per gm to 7 μg, respectively, there was an increased growth as shown in figures 2 and 3. Lepkovsky and Nielson ('42) reported a relationship of pyridoxine to tryptophane metabolism in rats. They suggest that normal tryptophane metabolism is regulated by pyridoxine. That pyridoxine is necessary for the formation of the coenzyme which functions in the tyrosine decarboxylation reaction of certain bacteria has been shown by Gunsalus, Bellamy and Umbriet ('44). Follis and Wintrobe ('45) find that a deficiency of pyridoxine and pantothenic acid in the diet of swine resulted in degeneration of the sensory neurons. While it has not been definitely proven that pyridoxine and pantothenic acid are essential in the diet of humans, experiments on other animals indicate that these vitamins have a function in animal metabolism.

Sure ('41) noted abnormalities in reproduction when calcium pantothenate was lacking in the diet. Nelson and Evans ('46) found that a deficiency of pantothenic acid in the diet on or before the day of mating always resulted in failure to reproduce or in abnormal young. The results reported in the present paper are in line with those of these investigators since offspring were obtained only from the mothers who received calcium pantothenate in the diet and the litters were defective and did not mature.

The quantities of pyridoxine and calcium pantothenate along with the other B-complex vitamins to be added to enriched flour in order to produce normal growth and normal young require further study and this work is being continued.

SUMMARY

Evidence has been presented to show that the supplementing of enriched flour with certain B-complex vitamins improves the nutritive value of the flour. Under the conditions of the experiments the addition of 1 μg of riboflavin per gm of enriched flour produced an increase in the growth rate of the animals. The addition of 1 μg of riboflavin and 1 μg of thiamine to the enriched flour stimulated even greater growth of

the rats. The addition to 10.3 μg calcium pantothenate and 3.5 μg pyridoxine per gm of enriched flour was beneficial, but a still greater increase in growth resulted when 20.5 μg calcium pantothenate and 7 μg pyridoxine per gm were added to the enriched flour.

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AMINO ACIDS REQUIRED TO SUPPLEMENT LINSEED PROTEIN FOR CHICK GROWTH

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Linseed meal has been shown to be detrimental to growing chicks when it is fed at levels of 4.5% or more of the ration (Bethke et al., '28; Ackerson et al., '38; Sherwood and Couch, '40; Slinger et al., '43; Hammond, '44; Heuser and Norris, '44; Heuser et al., '46). Since its nutritional value may be increased by a simple water treatment (Kratzer, '46; McGinnis and Polis, '46), apparently the detrimental action of linseed meal depends upon a toxic substance, not upon a serious amino acid deficiency in the protein. By using water-treated linseed meal as the sole source of protein in a chick ration, one can determine the adequacy of the protein in meeting the amino acid requirement of chicks without the interference of the toxic factor.

EXPERIMENTAL PROCEDURE

Commercial linseed meal was treated with water for 24 hours and dried according to the procedure outlined previously (Kratzer, '46). The composition of the basal diet in which the treated linseed meal was fed is given in table 1. The crude protein content of the diet was not determined for the first experiment, but in the second and third experiments the rations were found to contain 18.5 and 20.3%, respectively.

Day-old S. C. White Leghorn chicks were fed a practical starting ration for 11 days. At the end of this period they were divided into comparable groups of 8 chicks each and were fed the experimental rations ad libitum. They were housed in electrically heated batteries with wire floors. The trials were continued for 9, 8, and 7 days, respectively, in successive

TABLE 1

Minerals and vitamins contained in the basal diet.

BASAL MIXTURE	
Treated linseed meal:	
Trials 1 and 2	58 gm
Trial 3	61 gm
Minerals, vitamins and other organic supplements . . . as indicated below	
Glucose: to bring the total up to	100 gm

MINERAL SUPPLEMENTS	gm	VITAMINS AND OTHER ORGANIC SUPPLEMENTS	gm
Calcium gluconate	5.0	Soybean oil	3.0
Tricalcium phosphate	2.0	Fish oil (3000 A–400 D)	0.5
Dicalcium phosphate	1.5	Choline chloride	0.2
Sodium chloride	1.0	Cholic acid	0.1
Dipotassium phosphate	0.5	Inositol	0.1
Magnesium sulfate	0.3	Niacin	0.01
Potassium chloride	0.3	dl-alpha tocopherol acetate	0.001
Sodium silicate	0.2	Calcium pantothenate	0.001
Manganese sulfate	0.03	2-methyl-1, 4-naphthoquinone	0.001
Ferric oxide	0.02	Riboflavin	0.0005
Aluminum sulfate	0.025	Thiamine	0.0005
Copper sulfate	0.005	Pyridoxine	0.0004
Zinc sulfate	0.005	Folic acid	0.0001
Cobalt acetate	0.002	Biotin	0.00001
Potassium iodide	0.001		

experiments. With the exception of 1 chick which had been fed the basal ration with no amino acid supplements in the third experiment, there was no mortality. One control group fed stock mash was included in each experiment.

Amino acids were added to the basal ration as indicated in table 2. Those used were commercial preparations in the following forms: l (+)-arginine monohydrochloride, l(—)-

cystine, l(+)-lysine monohydrochloride, dl-methionine, and dl-tryptophane.

The growth data were calculated as per cent gain per day according to the following formula:

$$\frac{\text{total gain} \times 100}{\text{average body weight} \times \text{number of days}} = \text{per cent gain per day}$$

This criterion permits a better comparison of data from different experiments than is possible by the use of average gains.

TABLE 2

Growth of chicks fed a basal ration containing linseed protein supplemented with various amino acids.

SUPPLEMENT	LEVEL IN PER CENT OF RATION	GAIN BODY WEIGHT (% GAIN PER DAY)		
		Experiment 1	Experiment 2	Experiment 3
None	..	5.1	5.1	4.9
Arginine	0.2	5.1	5.5	..
Lysine	0.2	6.5	..	..
Lysine	0.4	..	7.0	7.1
Lysine	0.5	6.9	..	..
Methionine	0.2	5.1	5.0	..
Cystine	0.162	5.6	5.5	..
Tryptophane	0.1	5.6	5.3	..
Glycine	0.5	5.9	5.4	..
Lysine	0.5	..	..	..
Methionine	0.2	7.2	..	..
Lysine	0.4	..	..	..
Methionine	0.2	..	7.0	7.1
Lysine	0.5	..	..	..
Cystine	0.162	6.7	..	..
Lysine	0.4	..	..	..
Glycine	0.5	..	..	7.4
Lysine	0.4	..	..	..
Methionine	0.2	..	8.3	7.2
Glycine	0.5	..	..	..
Arginine	0.2	..	..	..
Lysine	0.4 [1]	..	..	..
Methionine	0.2	7.2	8.3	..
Cystine	0.162	..	..	..
Tryptophane	0.2	..	..	..
Glycine	0.5	..	..	..
Stock Mash	..	7.4	8.3	6.8

[1] Lysine fed at 0.5% in experiment 1.

RESULTS AND DISCUSSION

As table 2 shows, in the first experiment lysine produced a marked growth response, while the addition of the other amino acids did not give any appreciable increase in the growth rate. The combination of lysine and methionine was slightly better than lysine alone and was as satisfactory as the combination of the 6 amino acids tested. Since 0.5% lysine caused an increase in growth above 0.2% lysine, evidently the lower level does not adequately supplement linseed protein.

In the second experiment, lysine again proved to be the only amino acid to cause an appreciable increase in growth rate. The addition of glycine to the ration containing lysine and methionine, however, caused growth to increase from 7.0% gain per day to 8.3%. Since the crude protein of the diet used in this experiment was suboptimal, conceivably the addition of even a dispensable amino acid might stimulate a growth increase.

With the crude protein level raised to 20.3% in the third experiment, lysine supplementation resulted in a growth rate of 7.1% per day, whereas the combination of lysine, methionine, and glycine caused a growth rate of 7.2% per day. Methionine added to the ration containing lysine alone caused no increase in growth; and when it was added to the ration containing both lysine and glycine, there was a reduction of 0.2% per day. When glycine was added to a ration containing lysine, growth was increased by 0.3% per day; and when glycine was added to a ration containing lysine and methionine, the increase was 0.1% per day. For the chick, therefore, linseed protein is apparently adequate in methionine. Glycine is probably adequate, although slight gains of doubtful significance were observed after its addition. Lysine constitutes the principal amino acid deficiency in linseed protein.

Block and Bolling ('45) have found that linseed protein contains the following percentages of amino acids: arginine, 6.2; lysine, 2.5; methionine, 3.0; cystine, 1.9; and tryptophane, 1.9. For a protein to be complete for the chick with respect to these amino acids (Almquist, '42; Grau and Almquist, '43,

'44), it should contain arginine, 4.5%; lysine, 4.5%; methionine, 2.5%; cystine, 1.5%; and tryptophane, 1.0%. According to these values, lysine is the only amino acid in which one would expect linseed protein to be deficient for producing rapid growth of the chick.

Since linseed meal supplied all the protein in these tests, rather than protein that is merely supplementary to the cereal proteins of a practical ration, the level of linseed meal was greater than would ever be used in a practical ration. In every experiment, a diet containing linseed protein properly supplemented with amino acids produced as good growth as a practical chick starting ration. Thus the water treatment applied to the meal seems to have been entirely effective in destroying the growth inhibitor, and the principal limitation in the practical use of linseed protein lies in its lysine deficiency.

SUMMARY

1. Linseed meal treated with water to destroy its toxic principle was fed to chicks as the sole source of protein.

2. Lysine is the principal amino acid deficiency in linseed protein.

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AN INBORN CHARACTERISTIC DETERMINING THE RESPONSE OF CHICKENS TO A DIET FREE OF ANIMAL PROTEIN

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TWO FIGURES

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It was reported by Rubin and Bird ('46) that the feeding of concentrates prepared from cow manure stimulated the growth of chicks whose basal diet consisted largely of corn and soybean oil meal. The assaying of such concentrates was greatly facilitated by using the progeny of hens fed a similar deficient diet, since hens whose diets contained the growth-stimulating factor readily transmitted it to their chicks.

Further investigation has revealed that the response of chicks to the diet being studied is influenced not only by the diet of their dams but also by some unknown characteristic of the dams which is highly variable among the population studied. This variability is manifested in hatchability of eggs and in viability and growth of progeny. The present report is concerned with this variability which is of theoretical interest for its own sake and of practical interest because of its undesirable effect on the study of diets and dietary supplements.

METHODS AND RESULTS

Variation in response of hens to a diet inadequate from the standpoint of hatchability

The experimental diets used both in growth and in hatchability studies are summarized in table 1. They are identical

with the diets used in previously reported experiments (Rubin and Bird, '46; Bird et al., '46).

The hens were yearling crossbreds (Rhode Island Red × Barred Rock) and were mated to New Hampshire males. Just prior to the initiation of these studies they had been used in a hatchability experiment of approximately 10 months' duration and had been fed the basal diet or a slightly modified diet

TABLE 1

Composition of diets.

INGREDIENTS	GROWTH STUDIES	HATCHABILITY STUDIES	
	Basal mixture	Basal mixture	Positive control
	%	%	%
Ground yellow corn	38	57	78.3
Ground barley	20		
Alfalfa meal	3	5	5
Soybean meal	35	30	
Sardine meal			10
Butyl fermentation solubles (250 μg riboflavin/gm)	0.6	0.5	0.5
Limestone flour	1.0	2.3	2.0
Steamed bonemeal	1.5	4.2	3.2
Salt (96% NaCl, 4% Mn SO_4)	0.7		
Salt (94% NaCl, 6% Mn SO_4)		0.5	0.5
Iodized salt		0.2	0.2
Vitamin A and D oil [1]	0.2	0.3	0.3
Total	100.0	100.0	100.0
Added to the above			
Choline chloride	0.05		
Nicotinic acid hydrochloride	0.001		

[1] 2000 U.S.P. units of vitamin A and 400 A.O.A.C. units of vitamin D per gm.

similar in its effect on hatchability (Bird et al., '46). Some of the data accumulated in that experiment were used in the present investigation.

Individual hatchability records were calculated for 183 hens which had been housed in laying houses and fed the basal diet or a modification similar in its effect on hatchability. Individual records covering a period of 6 months were also calcu-

lated for 67 hens of the same breeding kept in laying cages on wire screen floors and fed the basal diet. All eggs were incubated except those produced during 4 non-consecutive weeks. Table 2 shows the distribution of the hens into groups characterized by low, intermediate, and high hatchability. The birds kept on wire floors were treated separately because it had been shown that cow manure fed with this basal diet was highly beneficial to hatchability, and it was thought that coprophagy might explain the superior performance of some of the birds kept in laying houses. However, the distribution of the hens kept on wire floors was not much different from that of the others and coprophagy would not appear to be a factor. It is surprising to note that a number of individuals

TABLE 2

Distribution of hens fed deficient diet according to hatchability of their eggs and type of cage floor.

HATCHABILITY RANGE IN % OF FERTILE EGGS	HENS ON CONCRETE FLOORS COVERED WITH LITTER		HENS ON WIRE FLOORS	
	No. of hens	% of total	No. of hens	% of total
0–70	80	44	33	49
70–85	68	37	17	25
85–100	35	19	17	25

maintained excellent hatchability during a period of 10 months although they received a diet that was quite inadequate for the majority.

It seemed possible that this variability might be due to differences in storage of the critical factor or to differences in ability to make adaptation to an unfavorable diet. In order to determine if there were any significant trends in hatchability during the course of the experiment, the hens characterized by low, intermediate, and high hatchability were divided into groups numbered 1, 2, and 3, respectively, and continued on the basal diet. After a lapse of 4 weeks all eggs were collected and settings made at weekly intervals as before. The hatchability records of the 3 newly constituted groups were pro-

jected back to the beginning of the previous experiment by combination of the records of the individuals for this period. The results, covering a period of 17 lunar months, are plotted in figure 1.

The consistency with which the 3 groups maintained their relative positions is striking. It would be difficult to explain the long-continued difference in hatchability between groups 1 and 3 on the basis of storage. Neither does adaptation to

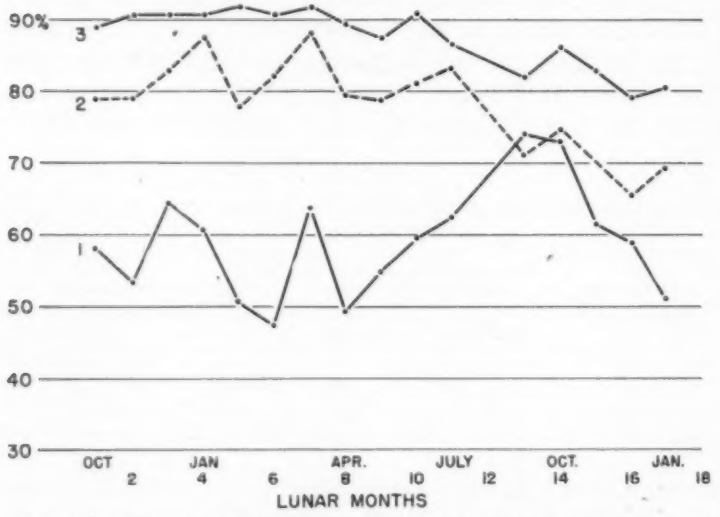

Fig. 1 Hatchability of fertile eggs of hens fed deficient diet. Groups segregated on basis of hatchability.

the diet seem a promising explanation since one would have to assume that the hens of group 3 accomplished this during the first month, those of group 2 made a partial adaptation during the same time but no subsequent improvement, and those of group 1 were incapable of making the adaptation. The sustained increase achieved by group 1 between April and September and the subsequent decline may have been seasonal although the increase came somewhat later than the usual seasonal increase.

Growth promoting effects of hen manure

Rubin et al. ('46) reported that the urine-free feces of hens were as effective as cow manure in promoting growth of chickens, even though the hens had been fed a diet that was low in the growth factor. The total excrement of hens was less effective presumably due to the presence of urinary products. Although the variation in response of the hens to the deficient diet was not related to coprophagy, it was thought that it might be due to differences in extent of synthesis of the critical factor in the digestive tracts of different hens. Accordingly the total excrement of groups 1 and 3 was collected, dried and fed to growing chickens as 5% of the basal diet during the

TABLE 3

Average weights of chickens at 6 weeks of age as affected by feeding 5% manure.

SOURCE OF MANURE	AVERAGE WEIGHT OF CHICKENS
	gm
Hens of group 1 fed basal diet	454
Hens of group 3 fed basal diet	463
Urine-free feces of a hen fed basal diet	521
Hens fed positive control diet	491
None (basal diet without manure)	413
Least significant difference (19 : 1 odds)	63

first 6 weeks of life. Comparable groups of growing chickens were fed the same level of urine-free feces of hens, and the excrement of hens fed the positive control diet which supported hatchability at optimal or near optimal levels. These supplements replaced 5% of corn. The results are summarized in table 3. They were reported in part in the paper by Rubin et al. ('46).

No material difference in growth-promoting properties was demonstrated between the excrements of groups 1 and 3. In fact, neither provided a significant stimulus to growth according to the usually accepted standards of significance. A significant stimulus was provided by the urine-free feces of

a hen fed the basal diet and by the total excrement of hens fed the positive control diet.

It was thought that the 3 groups of hens might differ in efficiency of feed utilization or in rate of passage of feed through the digestive tract, but this did not prove to be the case. The efficiency indices of groups 1 to 3, respectively, determined by the method described by Bird and Whitson ('46) were 1.01, 1.03, and 1.04. Coloring the feed with 5% of charcoal did not reveal any noteworthy differences in rate of passage of material through the tract.

Variation in response of progeny as related to maternal response

After redistribution of the hens into groups 1, 2, and 3 on the basis of hatchability, one entire hatch was used in an experiment to determine whether there was any relationship between the responses of dams and progeny when both were fed the deficient diets. The progeny of groups 1, 2 and 3 numbered 96, 49 and 58, respectively. In addition 66 chicks hatched from eggs of hens fed the positive control diet were used. These chicks were wing-banded and distributed among 7 pens of a brooder house so that each of 5 pens contained an approximately equal number of chicks from each group of hens. The remaining 2 pens contained a preponderance of chicks from the hens of group 1, with a few from group 3 and the hens fed the positive control diet.

The average growth curves to 6 weeks of age and the per cent of viability during the first week are shown in figure 2. Growth and viability showed the same trend as did hatchability. Within the 6 weeks' duration of this experiment the maternal influence showed no diminution, though all of the chicks were fed the same diet throughout. The hens of group 3 were able, in spite of their deficient diet, to maintain hatchability and viability of offspring at levels that compared favorably with the performance of the hens fed the positive control diet. However, the growth rate of their progeny was

decidedly inferior to that of the chicks from hens fed the positive control diet.

Since it had proved profitable to use the offspring of hens fed the deficient diet in assaying concentrates of the growth factor prepared from cow manure, it seemed worthwhile to determine if any further advantage would be gained by using only chicks from the hens most susceptible to the deficiency.

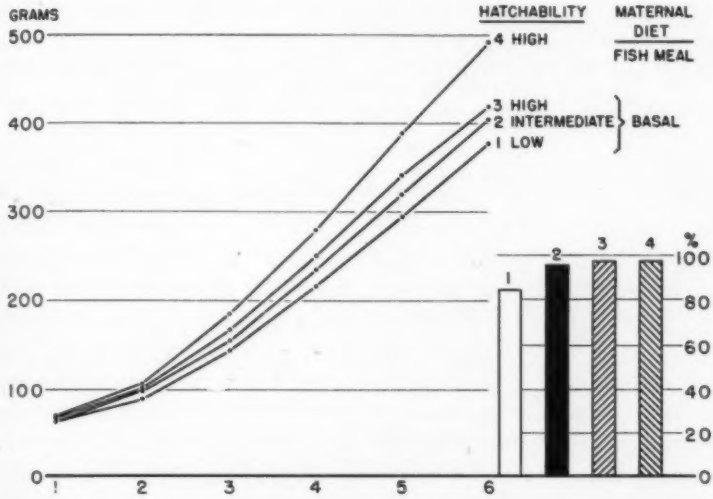

Fig. 2 Growth curves of chicks to 6 weeks of age and per cent viability (bar graphs) during first week, related to hatchability and maternal diet. Groups 1, 2, and 3 were progeny of dams fed basal diet and characterized, respectively, by low, intermediate, and high hatchability. Group 4 consisted of progeny of dams fed positive control diet containing fish meal.

It seemed possible also that such chicks might be useful in distinguishing between the effects of concentrates from manure and of other supplements. It has been reported that a growth stimulation is obtained when this diet is supplemented with calcium pantothenate and choline chloride (Bird and Rubin, '46) and when similar diets are supplemented with dl-methionine (Bird and Mattingly, '45). There is evidence,

however, that the concentrates do not owe their effect to these substances (Rubin and Bird, '46).

Two experiments were performed in which the basal chick diet and the modifications shown in table 4 were fed to chickens during the first 6 weeks of life. In experiment 1 there were in each pen 5 chicks from the hens of group 1, 12 chicks from hens of group 2, 8 chicks from those of group 3, and 8 chicks from hens fed the positive control diet. In experiment 2 each

TABLE 4

Effect of dietary supplements on growth of the progeny of deficient hens and hens naturally resistant to deficiency.

EXPERIMENT NO.	SUPPLEMENT TO BASAL DIET FOR CHICKS	AVERAGE WEIGHT OF 6-WEEK-OLD PROGENY OF: Group 1 hens (deficient) fed basal diet	Group 2 hens (intermediate) fed basal diet	Group 3 hens (resistant to deficiency) fed basal diet	Mixed hens fed positive control diet
		gm	*gm*	*gm*	*gm*
1	None	367	372	458	489
	0.05% acid ppt. of manure	...	511	505	532
	0.2% dl-methionine	434	451	478	541
	0.002% calcium pantothenate plus 0.1% choline chloride	393	427	501	554
2	None	331	326	472	
	0.2% dl-methionine plus 0.002% calcium pantothenate plus 0.1% choline chloride	422	405	515	

pen contained 11 chicks from the hens of group 1, 6 from group 2, and 5 from group 3. The results are shown in table 4. The figure for the progeny of the hens of group 1 fed the acid precipitate of manure extract was omitted because early mortality left too few chicks to be of any significance. The growth rates of the 3 kinds of chicks were approximately the same when all were fed the fraction prepared from manure, but this was not true when the other supplements were fed. Among the pro-

geny of the group 2 hens, the manure fraction was much superior to the other supplements, judged by growth response; among the progeny of group 3, its superiority was doubtful; and among the progeny of the hens fed the positive control diet the other supplements were at least as effective. Since, in the experiments summarized in table 4, the number of chicks fed the manure fraction was small, it may be worthwhile to point out that the results have been confirmed in numerous assay experiments in which the same fraction has elicited a maximal or near-maximal growth response in lots made up largely of the progeny of groups 1 and 2 (Rubin and Bird, '46; and unpublished data).

DISCUSSION

The results of these experiments make it clear that chickens vary widely with respect to their stores, at hatching time, of the dietary factor in question and with respect to some other characteristic which influences their ability to withstand deficiency. These 2 sources of variability combined with the variability expected in a basal diet composed of natural ingredients could account for a considerable difference in the results of supposedly duplicate experiments. In the study of the unknown factor involved here, it has proved to be profitable to use only chicks from hens fed a diet low in the factor and to eliminate the progeny of hens resistant to the deficiency, such as those of group 3. It has also proved quite essential to use in hatchability experiments only hens shown to be susceptible to the deficiency.

The variation in response to different supplements, illustrated in table 4, probably accounts for the inconsistent results of numerous experiments performed in this laboratory with chicks of uncontrolled source. In some experiments the growth response produced by methionine was as great as that produced by cow manure or by fish meal: in other experiments it was much less. It is difficult to account for the results summarized in table 4 without postulating that the unknown factor in manure, methionine and the combination of choline and

pantothenate are to some extent interchangeable for the "better" chicks. This might mean that the unknown factor is partially replaceable, and that the "better" chicks had received sufficient of it from their dams so that their remaining requirement was met by methionine or the combination of choline and pantothenic acid.

It is anticipated that future work will reveal whether or not the characteristic causing the variable response of hens to the deficient diet is inherited. This question cannot be answered on the basis of the evidence now available, since the relationship between growth response of progeny and hatchability response of dams might have been due to differences in the quantities of the essential factor stored in the eggs. There has been surprisingly little investigation of the inheritance of resistance and susceptibility to dietary deficiency. Different strains of rats have different vitamin D requirements according to Gowen ('36), different thiamine requirements according to Light and Cracas ('38), and different choline requirements according to Engel ('43), and differ with respect to efficiency of feed utilization according to Morris et al. ('33). Lamoreux and Hutt ('43) developed strains of White Leghorn chickens which varied in their requirement of riboflavin for growth. Davis et al. ('38) studied the response of 3 full sisters in each of 5 different families of White Leghorns to a diet low in riboflavin, and found that the families differed significantly with respect to hatchability of fertile eggs. However, Lamoreux ('38) reported that there was not a very close relationship between precentages of hatchability of fertile eggs produced by dams and daughters fed a diet low in riboflavin.

Whatever its basis may be, the relationship under discussion is a very close one, involving both maternal diet and maternal variability in response to diet and manifesting itself in hatchability, viability of progeny, and growth rate of progeny at least to the age of 6 weeks.

SUMMARY

Examination of the individual hatchability records of 183 hens which had been fed a diet containing no animal protein for a period of 11 lunar months revealed that 44% of the hens showed hatchability figures between 0 and 70%, 37% showed from 70 to 85% hatchability, and 19% showed from 85 to 100% hatchability. Further investigation revealed that the high hatchability maintained by the minority could not be explained on the basis of coprophagy, storage of the essential factor, or adaptation to an unfavorable diet.

Hens characterized by high and by low hatchability were not found to vary with respect to the growth promoting properties of their excreta or with respect to their efficiency of feed utilization.

The progeny of hens characterized by high, intermediate, and low hatchability, respectively, showed high, intermediate, and low viability and growth rate to 6 weeks of age. The effect of dietary supplements upon the growth of chickens varied with the maternal diets and with the ability of the dams to withstand dietary deficiency. This variation may be of considerable importance, especially in experiments in which the diets are variable because of their content of natural feedstuffs.

The variability among the hens is believed to have been inborn, as that of their progeny certainly was. Whether the variation of the progeny was congenital or hereditary cannot be stated on the basis of present evidence.

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SURFACE AREA AND METABOLISM OF GROWING GUINEA PIGS [1]

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TWO FIGURES

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This is one of a series of reports on the metabolism of growing laboratory and farm animals, including rats, chickens (Kibler and Brody, '42; '44) cattle, horses, and swine recently summarized by Brody ('45). As in the preceding reports, surface area of the skin and body weight raised to some power (W^b) are used as reference bases.

SURFACE AREA OF THE GUINEA PIG

The guinea pig has a rather regular shape so that it appeared fairly simple to determine the surface area from appropriate linear measurements on the living animals. The following average measurements (fig. 1) were accordingly made on each animal: length and width of both sides of each ear, length and circumference of the head, length and circumference of each leg, and the length and girth of the trunk. Total surface area was then computed as the sum of the areas of these separate parts.

To check the areas computed from the linear measurements a second method was employed on the same guinea pigs. The animals were sacrificed by the use of illuminating gas, dipped in shellac and allowed to dry. Their skins were then removed

[1] Mo. Agric. Exp. Sta. Journal Series No. 1024.

and outlined on paper. The paper outlines were cut out and weighed. The skin area was then computed from the weight of the paper outline and the conversion factor for area per unit weight of paper.

The surface area data obtained by each of these 2 methods on 19 guinea pigs ranging in body weight from 230 to 1150 gm were plotted separately (fig. 1) and the power or logarithmic equation $Y = aX^b$ was fitted to each set of data by the least

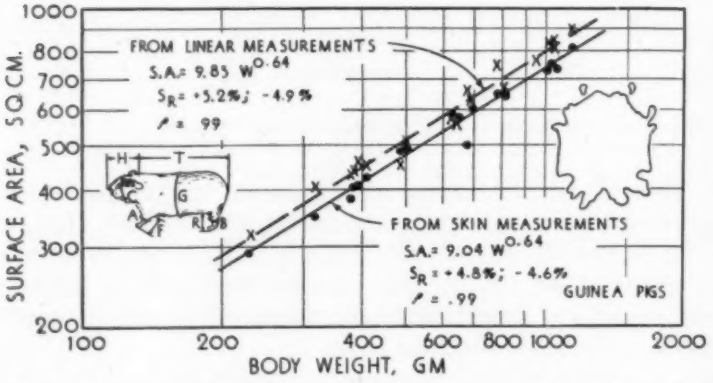

Fig. 1 Surface area of guinea pigs from linear measurements on living animals and from the measurement of the skins from the same animals. In the small figure at the left-hand side, lengths are indicated by H, head; T, trunk; F, front leg; R, hind leg. Circumferences are shown as C, head; G, trunk; A, front leg; B, hind leg. The length and width of each ear were also measured.

square method. For the linear measurements on the 19 living animals, the relation between surface area and body weight is given by the equation

$$S.A. = 9.85W^{0.64} \qquad (1)$$

For the measurements of the 19 shellacked skins, the relation is expressed by the equation

$$S.A. = 9.04W^{0.64} \qquad (2)$$

In both equations S.A. represents surface area in square centimeters, W represents weight in grams, and the exponent *b* indicates the relative rate of increase of surface and weight.

As b is 0.64 for both methods, the surface area increases 0.64 times as fast as the weight. The guinea pig, then, must change in form with increasing size, since — as can be shown by dimensional analysis — the exponent b would be $\frac{2}{3}$ if constant geometric similarity of form persisted during growth.

The variability of the data is about the same for both methods. The correlation coefficient [2] ρ is 0.99 for each equation (fig. 1). Two-thirds of the individual observations fall within approximately 5% of the respective equation lines as indicated by the standard errors of estimate,[3] S_R (fig. 1).

Equation 2, although parallel with equation 1, is lower presumably because of the shrinkage of the shellacked skins during drying. Therefore equation 2 was discarded and equation 1 from the linear measurement on the living animals was used for all subsequent computations.

METABOLISM

The metabolism (oxygen consumption) was measured in an eight-chamber closed circuit, volumetric apparatus (Kibler and Brody, '42). Detailed descriptions of similar multichamber volumetric appartus have been reported by Kleiber ('40), Winchester ('40), and Brody ('45, p. 325).

The measurements were made at thermoneutrality (about 30°C.). Corrections for activity were unnecessary as the guinea pigs were usually very quiet. Data were obtained during rest on each of the same 4 male and 4 female guinea pigs for the following conditions and periods: (1) non-fasting (birth to 1 year); (2) fasted for 24 hours (after 1 month of

[2] If the surface area and body weight data were perfectly correlated, the value of ρ would be 1.00. The value of 0.99 found for these data indicates a high degree of correlation that would have a probability of occurrence by chance of less than one in a hundred.

[3] $+S_R$ and $-S_R$, the standard errors of estimate, indicate the percentage range about the fitted line that includes $\frac{2}{3}$ of the data. For example, table 1 shows that a 400-gm guinea pig has a surface area of 456 cm², and figure 1 shows $+S_R = 5.2\%$ and $-S_R = 4.9\%$. The surface area derived from similar measurements on other 400-gm guinea pigs would not be expected (more than one time in three) to exceed 456 cm² by more than 23.7 cm² (0.52 × 456) or fall below 456 cm² by more than 22.3 cm² (.049 × 456).

age to 1 year); and (3) fasted 40 to 48 hours (after 5 months of age to 1 year).

A statistical test of the covariance of metabolism and body weight failed to establish a significant difference between the oxygen consumption values on the 24- and 48-hour fasts. Therefore all fasting data were combined and designated "basal metabolism." The "resting metabolism" data, including the calorigenic action of the feed, and the "basal data" are summarized in table 1.

During growth less extensive changes occurred in the metabolic rate per unit area in the guinea pig than had been found, for example, in the rat. In the data for the guinea pig (table 1) the lowest such value is 640, occurring in the ninth month, and the highest is 846, occurring about the sixth week, a range of only 206 kilo-cal. per square meter per day. In the data for the rat (Kibler and Brody, '42) a low value of 456 was found at 1 day after birth and a high value of 1241 at 45 days of age, a range of 785 kilo-cal. per square meter per day. These differences between species are undoubtedly due to the greater physiological maturity at birth of the guinea pig than the rat.

The detailed changes in metabolism per unit of surface area with increasing age and weight are shown in table 1 for both resting and basal conditions. In general the highest values occurred between 6 and 12 weeks of age, when the body weights ranged between 200 and 400 gm. The lowest values were reached between 8 and 12 months of age at body weights between 700 and 800 gm. A striking feature of figure 2 is that the female group of guinea pigs had a consistently higher metabolic rate per unit of surface area than the male group for all ages and conditions.

The surface area base is very useful for studying age changes in metabolic rate and for equalizing metabolic rates between mature animals of different size, but metabolic rate per animal can be predicted more accurately and conveniently from the relation between total metabolism C (kilo-cal. per day) and body weight W (gm) expressed by the equation

TABLE 1

Growth and metabolism of guinea pigs.

AGE PERIOD	FEMALES					MALES				
	Average per guinea pig					Average per guinea pig				
	No. observations	Body weight	Surface [1] area	Cal.[2] per day	Cal./m² per day	No. observations	Body weight	Surface [1] area	Cal.[2] per day	Cal./m² per day
		gm	*cm²*				*gm*	*cm²*		
			Resting					Resting		
Week										
B–1	14	98	185	15.1	816	4	104	192	15.5	807
1–3	43	131	223	16.8	753	43	134	226	16.4	726
3–5	39	186	279	22.3	799	30	189	282	22.1	784
5–7	33	259	345	29.2	846	36	258	344	27.1	788
7–9	18	314	390	32.1	823	22	296	376	30.0	798
9–11	15	380	441	35.9	814	15	386	445	35.7	802
11–13	29	400	456	38.2	838	15	411	464	36.7	791
Month										
3–4	51	483	514	42.1	819	43	507	530	40.6	766
4–5	62	564	568	46.7	822	31	596	588	45.1	767
5–6	54	609	596	47.6	799	36	676	638	47.3	741
6–7	45	655	625	47.5	760	31	722	665	46.4	698
7–8	44	687	644	47.8	742	31	723	666	44.8	673
8–9	29	789	704	52.6	747	26	710	658	44.9	682
9–10	19	861	744	58.5	786	19	790	704	49.6	705
10–11	30	873	751	56.3	750	30	765	690	46.6	675
11–12	32	814	718	55.9	779	21	777	697	49.4	709
			Basal					Basal		
Week										
3–5	5	172	266	19.8	744	8	177	270	20.0	741
5–7	5	241	330	25.8	782	7	242	330	25.0	758
7–9	7	281	364	29.7	816	3	260	346	27.0	780
9–11	5	356	423	33.2	785	6	372	435	32.8	754
11–13	4	394	451	38.2	847	2	390	448	38.5	859
Month										
3–4	9	447	489	39.0	798	7	440	484	38.7	800
4–5	17	518	538	42.8	796	8	582	579	41.9	724
5–6	18	588	583	43.9	753	12	653	624	45.8	734
6–7	23	634	612	44.2	722	16	688	645	45.0	698
7–8	27	624	606	42.4	700	17	698	651	42.9	659
8–9	12	755	684	45.8	670	12	684	642	41.1	640
9–10	10	784	701	50.2	716	10	737	674	44.2	656
10–11	22	807	714	50.6	709	18	739	675	44.7	662
11–12	31	782	700	50.5	721	18	734	672	45.7	680

[1] Surface area was computed from equation (1) in text.

[2] The heat production was calculated on the assumption that 1 liter of oxygen has a heat equivalent of 4.9 Cal. for normally fed guinea pigs and 4.7 Cal. for guinea pigs under basal conditions.

$C = aW^b$. When this equation is fitted to data for growing animals, however, the data may have to be divided into 2 or more segments (fig. 2), since the exponent b apparently changes with growth rate (Kibler and Brody, '42).

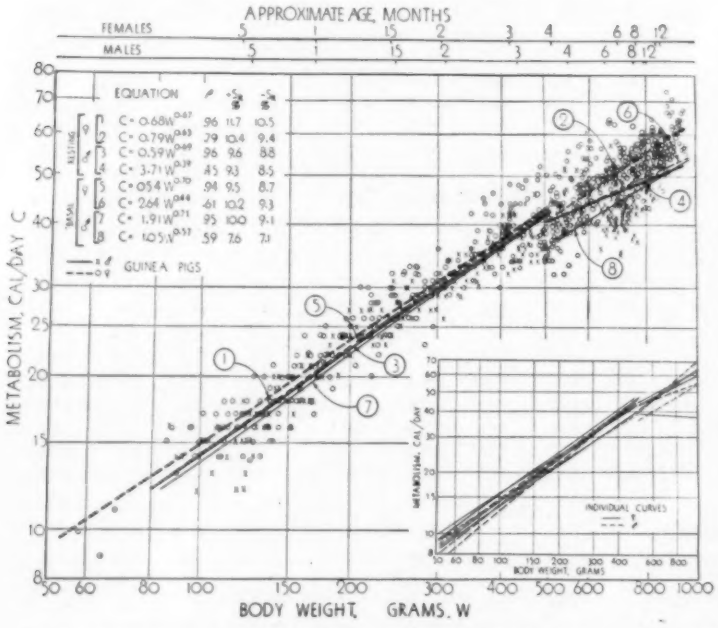

Fig. 2 Metabolism as a function of body weight in growing guinea pigs (individual observations plotted on logarithmic paper) with average age given on upper axis. Equations and statistical constants are tabulated for the male and female groups for resting and basal metabolism, but only the resting data are shown to avoid confusion. Separate curves for individual guinea pigs are shown in the inset in the lower right-hand corner.

In figure 2 the resting and basal metabolism data are plotted against body weight on logarithmically-divided paper. For the range of 50 to 500 gm body weight the equation $C = aW^b$ fits very well, the coefficients of correlation averaging 0.96 for the different curves. The 4 equation lines representing the resting and basal data for male and female guinea pigs are

nearly parallel as the values of exponent b vary only from 0.67 to 0.71. Above 500 gm body weight the distribution of the data is more erratic and the differences between the curves are more pronounced. The standard error of estimate, S_R, for all the metabolism data is about 10%, which is close to the values previously reported for other species. For given body weights, the fitted equation lines are higher for the female group than the male group for all data.

DISCUSSION

The lack of agreement on a reference base for equalizing metabolic rates between animals of different size is well known. It should be emphasized, however, that qualitative as well as quantitative differences appear when different bases such as body surface area, simple body weight or body weight raised to a power are used. For example, the metabolism per unit area for the guinea pig rises slightly for a few weeks after birth and thereafter declines; but the metabolism per unit weight declines from birth on. Since there is no general agreement on the reference base for energy metabolism, our results, as in the past, include data (table 1 and fig. 2) on body weight and metabolism per animal as well as metabolism in terms of unit area, and the relation between metabolism and weight raised to a power.

SUMMARY

Data are reported on metabolism (oxygen consumption) in relation to body weight, age, and surface area for the same 4 male and 4 female guinea pigs from birth to 1 year of age. The female group had a higher metabolic rate than the male group for the entire period of measurement. The average "resting" metabolism for both groups rose from 750 Cal. per square meter per day at 2 weeks after birth to 820 at 10 weeks and then declined to 720 at 7 months. Similar average "basal" values reached a maximum of 810 Cal. per square meter per day at 8 weeks and a minimum of 670 at 8.5 months.

From surface area measurements on 19 guinea pigs, ranging in weight from 230 to 1150 gm, the relation between surface area and weight was found to be expressed by the equation

$$S.A. = 9.85W^{0.64}$$

where S.A. is area in square centimeters and W is weight in grams.

We similarly related total metabolism to body weight by the same type of power equation and found that for guinea pigs weighing less than 500 gm, the metabolism increased more rapidly than surface area relative to increasing body weight (the exponent exceeded 0.64), and that for animals weighing more than 500 gm, the metabolism increased less rapidly than surface area with increasing body weight.

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ON THE GROWTH-PROMOTING FACTOR FOR RATS PRESENT IN SUMMER BUTTER

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ONE FIGURE

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PART I — CONFIRMATION OF ORIGINAL FINDINGS

Boer and Jansen ('41) showed that summer butter contains a growth-promoting factor for rats distinct from vitamin A, vitamin D, and the essential fatty acids. The results agreed in substance with those of Schantz and his co-workers ('40) although there were certain differences between our results which will be dealt with in a later publication. Euler and his co-workers ('42, '43) reported their inability to repeat our experiments; their work will be discussed below. We have further demonstrated (Boer et al., '44) that by treating the fatty acids of summer butter with suitable adsorbing agents the growth-promoting factor can be removed from the saponifiable fraction.

Experimental

In the experiments to be described 130 male rats, 4 weeks old, were each fed 1 of 8 different diets, the basal part of all diets being, by weight, as follows: wheat flour 72 parts, casein 5 parts, yeast 10 parts, and salt mixture 3 parts. The 8 different experimental diets were made by adding to 90 gm of this mixture 10 gm of a fatty fraction which it was desired to test. The additions for successive diets were as follows:

(a) Ten per cent of the glycerol esters of the fatty acids of summer butter plus the unsaponifiable part of 2 gm of summer butter daily. The glycerol esters were prepared by heating the dried fatty acids with an equivalent quantity of anhydrous glycerol at a temperature of 150°C.

(b) Ten per cent of the glycerol esters of the fatty acids of summer butter plus 2 I.U. of vitamin D (as calciferol) and 50 μg of β-carotene daily.

(c) The same esters as under heading (a), after adsorption of the esters on fuller's earth, plus the unsaponifiable part of 2 gm of summer butter daily.

(d) The same esters as under (c) plus 2 I.U. of vitamin D (as calciferol) and 50 μg of β-carotene daily.

(e) Ten per cent peanut oil plus the unsaponifiable part of 2 gm of summer butter daily.

(f) Ten per cent of peanut oil plus 2 I.U. of vitamin D (as calciferol) and 50 μg of β-carotene daily.

(g) Ten per cent of peanut oil after adsorption on fuller's earth, plus the unsaponifiable part of 2 gm of summer butter daily.

(h) Ten per cent of peanut oil after adsorption on fuller's earth, plus 2 I.U. of vitamin D (as calciferol) and 50 μg of β-carotene daily.

The growth experiments were continued for a period of 10 weeks, with weekly weighings. We have considered especially the weights after 4 weeks and after 10 weeks, first calculating the means in such a way that only litter mates were compared, and secondly making calculations for the total number of rats on each diet. Also, we have tabulated the mean of all the rats fed diets containing summer butter esters and the mean of all the rats on diets containing peanut oil, for each of the weeks from the fourth until the tenth.

Comparison of the litter mates after the fourth week

The differences between the weights of rats on the diets containing peanut oil and those of rats on corresponding diets containing the esters of fatty acids of summer butter are

very small (see table 1). No increase in weight was noticed due to the unsaponifiable fraction of butter or to the mixture of calciferol and carotene which was administered. No difference in weight was observed between the rats receiving the glycerol esters of summer butter whether they were treated by adsorption upon fuller's earth, or were left untreated.

Comparison of litter mates after the tenth week

The difference between the effect of the summer butter esters and that of the peanut oil is evident for all comparable diets (see table 1). In 18 of the 27 pairs of litter mates the

TABLE 1

Growth of rats demonstrating the presence of a growth factor in summer butter.[1]

NUMBER OF RATS	GROUP I	GROUP II	GROUP III	GROUP IV	Groups I, II, III and IV combined.	GROUP V	GROUP VI	GROUP VII	GROUP VIII	Groups V, VI, VII and VIII combined.
	19	19	16	18		11	16	14	15	
Week										
4	99	99	97	97	98	97	89	89	87	90
5	122	123	119	120		118	111	110	109	
6	145	145	142	141	143	136	127	131	127	130
7	160	155	161	158	158	148	141	145	142	144
8	176	171	172	169	172	163	149	158	150	154
9	186	181	180	177	181	174	158	168	162	165
10	196	190	189	183	190	185	165	177	169	173

[1] Mean weights of rats by groups at the end of each week from the fourth to the tenth week of the experiment. Dietary supplements for the different groups:

Group I. Esters of the fatty acids and unsaponifiable part of summer butter.
Group II. Esters of the fatty acids of summer butter and calciferol plus carotene.
Group III. Esters after adsorption and unsaponifiable part of summer butter.
Group IV. Esters after adsorption and calciferol plus carotene.
Group V. Peanut oil and unsaponifiable part of summer butter.
Group VI. Peanut oil and calciferol plus carotene.
Group VII. Peanut oil after adsorption and unsaponifiable part of summer butter.
Group VIII. Peanut oil after adsorption and calciferol plus carotene.

growth of the rat receiving summer butter esters is better — often much better — than the growth of the litter mate receiving peanut oil. In 5 cases, the members of a pair grew equally well and in only 4 instances out of 27 was the growth better on the diet containing peanut oil. In litter mate comparison there was no significant difference between the rats receiving the unsaponifiable parts of butter and those receiving a mixture of calciferol and carotene. No significant change in the growth promoting power was obtained upon treatment of either summer butter or the peanut oil with fuller's earth as an adsorbing agent.

Comparison of whole groups after the tenth week

The rats were therefore considered as 2 groups: the first group contained all those rats which had received the summer butter acids or esters whether treated or untreated, and the second group contained all the rats which had received peanut oil whether treated by adsorption or not. The data are presented in table 1, where the body weights after 4, 5, 6, 7, 8, 9, and 10 weeks are given. After 10 weeks the mean weight of rats on the esters of the fatty acids of summer butter is 190 gm (column 6) as compared with a mean weight of rats on peanut oil of 173 gm (last column). The question arises, whether this difference of 17 gm in the mean weight of the 2 groups of rats is significant. It must first be considered, whether the sub-groups which have been combined together to make the larger groups comprise homogeneous material, statistically speaking. When the distribution of growth figures within each group is considered, there is no evidence of departure from statistically normal distribution, and therefore we are entitled to conclude that each group is statistically homogeneous. We are also entitled to compare the means of the 2 groups, and it appears that the difference of 17 gm is significant, since it is 4.6 times as great as the standard error of the difference between the 2 means. We are therefore justified in concluding that after 10 weeks there is a highly

significant difference between the weights of rats receiving summer butter acids as compared with those of rats receiving peanut oil. This difference in weight can only be attributed to the esters of summer butter.

Our data also suggest but do not establish conclusively from a statistical standpoint, that the presence of the unsaponifiable part of summer butter produces a better growth than the mixture of calciferol and carotene. A further investigation of this point is under way. It is necessary to consider now why Euler and his co-workers were not able to reproduce our results. In our opinion the explanation lies in the fact that these workers did not exactly reproduce our experimental conditions. In their experiments butter which was obtained from milk collected during March was used. According to our information, in Sweden the cows are not yet put out to pasture in the month of March. They receive at that time foodstuffs not containing fresh grass. In our observation it has been found that only summer butter (by definition butter from cows receiving fresh grass) possesses the property of promoting growth as described above. The experiments of Euler and associates were not strictly comparable with our earlier experiments, because they were not carried out with what we have defined as summer butter. A rational explanation is therefore provided to account for their inability to reproduce our experimental results.

Summary

1. Confirmation has been obtained of the growth-promoting action of the glycerol esters of the fatty acids of summer butter. An explanation has been provided of the inability of Euler and his co-workers to confirm our earlier results.

2. The treatment of summer butter with fuller's earth as an adsorbing agent does not always remove the growth-promoting factor from the glycerol esters of the summer butter acids.

3. These experiments suggest but do not conclusively establish that the unsaponifiable part of summer butter also

has a growth-promoting action apart from the action of vitamins A and D.

PART II — THE INFLUENCE OF ADSORPTION AND HYDROGENATION ON THE GROWTH-PROMOTING FACTOR OF SUMMER BUTTER

In part I we have shown that treatment of the glycerol esters of the fatty acids of summer butter with fuller's earth as an adsorbing agent did not always remove the growth-promoting factor. When adsorption treatment did produce a difference in growth, as seen earlier (Boer and Jansen, '42), this difference was never great. Further experiments have therefore been made to test the effect of adsorption upon the growth-promoting power and also to test the effect of hydrogenation of summer butter upon its growth-promoting factor.

Experimental

The summer butter was treated with an adsorbing agent in the following way: the summer butter fat was mixed with 10% by weight of dried fuller's earth, stirred for half an hour at a temperature of 80°C., and then filtered with suction through a Buchner funnel. Another portion was hydrogenated, the hydrogenation being performed at 35°C. with Raney nickel catalyst, by the method of Kentie and Nauta ('45).

A feeding experiment was then carried out with 3 groups of rats to compare the effects on growth of filtered summer butter treated with the adsorbent and of hydrogenated summer butter with that of the untreated butter. The groups contained 11 or 12 rats each, and were fed the same basal diet as was used in the experiments of Part I. Litter mates were distributed among the experimental groups as evenly as possible.

The mean results are shown in table 2. After 3 weeks on the experimental diets the untreated summer butter (see Group I) had already produced a greater growth than the filtered summer butter (13 gm — Group II). This difference

was not due to the unsaponifiable part of the butter since this part had been added to all the diets. The difference increased each week for 11 weeks of the experiment; in the last week there was a slightly greater growth due to the treated butter. In table 3 is shown the individual growth of

TABLE 2

The comparative growth promoting action of untreated summer butter (Group I), filtered summer butter after heating and stirring with fuller's earth (Group II), and hydrogenated summer butter (Group III).

WEEK OF EXPERIMENT	BODY WEIGHT IN GRAMS AT END OF WEEK		
	Group I	Group II	Group III
3	95	82	73
4	127	110	98
5			
6	165	143	124
7	178	153	127
8	192	167	146
9	203	176	158
10	211	182	169
11	220	189	174
12	231	202	187

TABLE 3

The growth of individual rats after 12 weeks of the experiment, group I given untreated summer butter, group II given filtered summer butter after heating and stirring with fuller's earth, and group III given hydrogenated summer butter. The body weights are for individual rats after 12 weeks on experiment. Rats on same line are littermates.

LITTER	GROUP I	GROUP II	GROUP III
	gm.	*gm.*	*gm.*
1	241	175	190
1	221	195	193
2	234	199	174
3	225	209	171
7	247	207	185
7		184	204
9	242	242	202
11	241	220	185
11	207		209
12	232	197	185
13	219	192	158
Mean	231	202	187

the animals for the 12-week period. In all comparisons except one the growth on untreated summer butter was better — in many cases considerably better — than the growth on the filtrate of the same butter after treatment with fuller's earth. It may be taken that the difference between the means is real and not to be explained as due to accidental deviation of the growth of one or a few rats in the group. The difference between the means of the untreated summer butter and the filtered summer butter groups amounts to more than 3 times the standard error of these means and therefore is highly significant.

In the same way it appears from tables 2 and 3 that the growth maintained by hydrogenated summer butter is less than that which is maintained by the whole summer butter. The difference is again evident by the end of the third experimental week and increases during the following weeks until at the end of the experiment the rats receiving the summer butter have on the average gained 51 gm more than those receiving the hydrogenated summer butter. This difference is also highly significant as it amounts to 6 times the standard error of the difference between the means. Figure 1 emphasizes these differences graphically. We are therefore justified in concluding that the hydrogenation of summer butter removes its growth-promoting action. As a corollary it follows that the growth-promoting factor must be an unsaturated substance or a combination of unsaturated substances. This conclusion appears to contradict the finding of Schantz et al. ('40) that the growth-promoting factor of summer butter is a saturated compound.

An objection to the above experiments might be made, that the hydrogenated butter fat is less well absorbed from the intestinal tract because it is a hardened fat. To meet this objection we determined the fat content of the feces of the rats of the 2 groups, and found that the absorption of the normal summer butter is about 3% less than that of the hydrogenated product. As the fat-content of the diet is 10%, it follows that the rats receiving the hydrogenated summer

butter fat absorbed 0.3% less of the total diet than the rats on normal summer butter, a difference which cannot explain the great difference in growth rate.

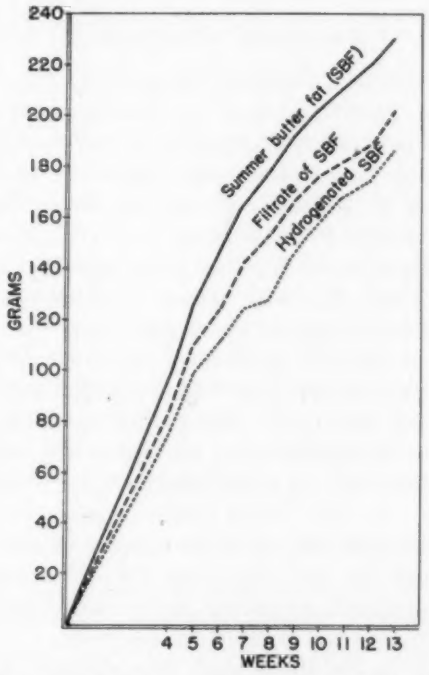

Fig. 1 The mean growth of rats receiving treated and untreated summer butter fat.

Summary

1. When summer butter fat is treated with fuller's earth the filtrate loses the growth promoting action of the original summer butter. The difference in growth is highly significant.

2. The growth produced by hydrogenated summer butter fat is quite inferior to that supported by the unchanged summer butter. This difference also is highly significant, and was shown not to be explained by different rates of absorption

of the 2 fats. Hydrogenation destroys or changes the growth-promoting factor in summer butter.

PART III — ON THE GROWTH-PROMOTING ACTION OF INDIVIDUAL SUMMER BUTTER ACIDS OBTAINED BY FRACTIONAL DISTILLATION

In our earlier experiments reported in Part I above we compared the growth of rats on summer butter with the growth when peanut oil was added to the control diet. The difference in growth between the different groups of rats appeared in 3 to 4 weeks; it was not great but increased continuously during the following weeks. The slowness with which the difference became manifest when peanut oil was used is a definite drawback which accompanies the use of this oil. In some of our earlier experiments we had obtained the impression that the growth of control rats on olive oil diminished at an earlier time than on peanut oil, so that with the shortage of peanut oil which developed during the war we changed to the use of olive oil. Later on, when this also became unobtainable, we tried rape-seed oil which gave even better results. It was found that the growth on rape-seed oil and on olive oil not only diminished at an earlier time than on peanut oil, but also, that the difference in growth between the animals receiving butter and the control rats was greater than when peanut oil was used.

The growth in the third week was already distinctly less with olive oil in the diet than when summer butter was used. After the third week the difference increased much more than when peanut oil was given instead of olive oil. For testing the various fractions prepared from summer butter the following procedure was devised. For the first 3 or 4 weeks of the experiment, all the rats received a basal diet containing olive oil (or later rape-seed oil). The litters were then divided between the 3 types of diet to be used in the latter part of the experiment, namely, the diet containing the summer butter, the diet containing the olive oil and that containing the olive oil with added fractions to be tested. It was found that

with this procedure involving the use of olive oil we were able to demonstrate a difference in growth with summer butter within 2 weeks.

Fractionation of the fatty acids of summer butter

The butter fat was saponified and the methyl esters of the fatty acids were prepared. These esters were subjected to fractional distillation at low pressures, by the method of Podbilniac, using a distillation column 2½ m in length.[1] The lower fractions up to fraction 10 (presumably C_{18}) were probably each fairly pure esters of a single acid (table 4). The higher boiling fractions were not esters of a single acid, but their fractionation served satisfactorily for a preliminary separation of active from inactive material. Each fraction was saponified and then esterified with glycerol at 150°C. (This was impossible in the case of butyric and caproic acids, whose boiling points were too low. These 2 acids were therefore esterified with ethanol.) A summary of the fractionation procedure is given in table 4.

Growth experiments with the different fractions

In the first experiment the first 4 fractions of table 4 were tested. Eighty rats, male and female, were distributed as equally as possible with regard to litter mates among 5 groups. Each group received the basal diet with an added 10% of fat. The first group received 10% olive oil. The succeeding 4 groups received fractions described under numbers 1 to 4 in table 4. In groups II, III and IV that amount of the fraction present in 20 gm of butter plus olive oil to bring the weight up to 10 gm total fat was added. The rats were maintained on the different diets for 1 week, and at the end of this time there was no difference in the growth rate of the different groups. Further experiments described below

[1] We are very much indebted to Prof. D. J. Coops who kindly permitted us to use the fractionating column in his chemical laboratory of the Free University of Amsterdam.

indicate definitely that when the growth-promoting factor is present the difference in growth between groups receiving the factor and those receiving only olive oil or rape-seed oil appears within the first week of the experiment. When there is no difference in growth found during the first week we are therefore entitled to conclude that the growth-promoting factor very probably is absent from the fraction under test.

TABLE 4

Fractional distillation at 15 mm of the methyl esters of the fatty acids of summer butter.

FRACTION NO.	WEIGHT IN GM	MOLECULAR WEIGHT	IODINE-VALUE	PRINCIPAL COMPOUNDS	DESCRIPTION
1	14		1.30	butyric acid	liquid
2	26.5	128.9	6.00	caproic acid	liquid
3	21.8	159.2	3.22	caprylic acid	liquid
4	40.6	185.7	16.36	capric acid	liquid
5	52.2	215.3	9.61	lauric acid	liquid
6	171.0	244.6	9.18	myristic acid	liquid
7	33.8	253.8	9.68	mixture	liquid
8	360.4	263.3	10.27	mixture	solid
9	93.5	279.1	35.99	mixture	half-solid
10	189.0	298.5	72.81	mixture	liquid
11a	253.4	293.4	73.44	mixture	half-solid
11b	172.3	291.6	66.80	mixture	half-solid
12	88.8	293.5	45.52	mixture	half-solid
14	24.7	277.2	73.46	mixture	solid
15	10.0	265.2	68.44	mixture	solid
16	23.3	283.3	61.44	mixture	solid
residual	54.5		38.51	mixture	solid

The conclusion is therefore justified that the growth promoting action of summer butter is not caused by the volatile fatty acids it contains, as is sometimes supposed on account of their presence in butter but not in other fats. The next 3 fractions containing chiefly lauric, myristic and tetradecenic acids, respectively, were similarly tested with negative results. In a third experiment fraction 8, fraction 9, and the heated glycerides of summer butter acids were examined with the results shown in table 5. The heating experiment

was designed to subject the natural glycerides of summer butter to the same high temperature as was used in preparing the glycerol esters after fractionation of the natural fat of the butter. The object was to test the effect of the heating upon the growth promoting factor present in the butter in order to determine whether it was stable to such a degree of heat. The growth experiment lasted 3 weeks and from table 5 it can be seen that fraction 8 contained the growth-promoting factor but that fraction 9 did not. The

TABLE 5

Growth of rats after 3 weeks on the basal diet with different added fats: group I, olive oil; group II, olive oil plus fraction 8 (table 4); group III, olive oil plus fraction 9 (table 4); group IV, heated summer butter fat.

LITTER FROM WHICH MATES WERE TAKEN	WEIGHT OF RATS			
	Group I	Group II	Group III	Group IV
	gm.	*gm.*	*gm.*	*gm.*
1	66	77	68	76
2	27	75	60	65
8	58	87	51	51
7		81	41	71
9	50	57	61	79
10	80	101	89	91
13	64	70	65	70
14	51	72	65	59
4	76	87		
6	34	65		
12	64	58		
mean	57	76	63	70

last column of the table shows that the growth on the esters exposed to a high temperature is distinctly better than the growth on olive oil. Thus we might also conclude that a high temperature does not necessarily destroy the factor wholly, although we cannot be sure that a partial destruction has not taken place. In order to extend the evidence for the existence of the growth-promoting factor present in fraction 8 we have repeated the experiment twice, first over a period of 2 weeks and then over a period of 1 week. The results

are summarized in table 6, and it is clear in each case with the shorter experiment that there is good evidence for the existence of the growth-promoting factor in this fraction.

TABLE 6

Growth of rats after 2 weeks and after 1 week on the basal ration with the addition of olive oil alone as compared with that of olive oil plus the active fraction of summer butter (group I — olive oil; group II — olive oil plus fraction 8 in table 4).

LITTER FROM WHICH MATES WERE TAKEN	WEIGHT IN GM	
	Group I	Group II
	Weight after 2 weeks on diet	
4a	38	40
6a	16	32
3a	22	42
2a	32	64
2b	33	54
3b	17	66
3b	38	45
4b	30	42
1b	10	38
mean	26	49
	Weight after 1 week on diet	
1	31	22
2	11	31
8	23	38
9	21	22
10	33	38
13	29	30
14	22	29
4	24	38
6	22	34
12	27	22
4a	21	18
6a	17	16
3a	4	22
2a	14	40
mean	21	29

Summary

1. By using olive oil or rape-seed oil and feeding all the rats in an experiment for the first 3 weeks with the olive oil diet, before dividing them into groups which are to receive the different fractions, the test for the presence of the growth-promoting factor is improved so that it can be completed in 1 or 2 weeks.

2. We have fractionated the methyl esters of the fatty acids of butter and after replacing the methyl groups by glycerol residues, we have administered the different fractions to various groups of rats in the growth tests.

3. The fractions up to C_{18} acids did not contain the growth-promoting factor, but we have succeeded in isolating a fraction beyond the C_{18} acid that did contain the growth-promoting factor.

PART IV — ISOLATION OF THE GROWTH-PROMOTING FACTOR PRESENT IN THE FATTY ACIDS OF SUMMER BUTTER

In order to account for the apparent contradiction between our results reported above, and those of Schantz et al. ('40) we have made the following working hypothesis. The growth-promoting factor is an unsaturated substance containing 18 C-atoms, which, after Twitchell separation is found in the so-called saturated fatty acid fraction. It is distinct from oleic, linoleic and linolenic acids. The only known acid which satisfies the above conditions is vaccenic acid, which has been described by Bertram ('28), and identified by him as Δ-11, 12 elaidinic acid.

Separation of vaccenic acid from the fatty acids of summer butter

The fatty acids were first separated according to the method of Twitchell ('21) and the solid unsaturated fatty acids present in the fraction designated the saturated fatty acids were isolated as described by Bertram.

Four hundred and seven gm of fatty acids derived from summer butter were dissolved in 500 ml of 96% ethanol, and to the warm solution was added a boiling solution of 130 gm of lead acetate and 1 liter of ethanol, and the mixture allowed to stand for 48 hours for crystallization of the lead soaps. The precipitated lead soaps were filtered with suction and then washed out with 250 ml of ethanol. The solid lead soaps were crystallized once more from 2 liters of ethanol, and then were boiled with hydrochloric acid (in the ratio of 1 to 1), dissolved in ether and washed out. The iodine value (Hanus) of the solid fatty acids obtained upon distillation of the ether was 8.8.

These solid fatty acids were warmed to liquefy them and added to a warm solution of 250 gm of mercuric acetate in 200 gm of acetic acid and 800 ml of 98% methanol. After standing for 24 hours the precipitated mercury salts were filtered with suction, and the filtrate cooled and at the same time 175 ml of concentrated HCl added. The acids which separated were taken up in petroleum ether, and the solution was washed with water and filtered. The petrol-ether was removed by distillation and left behind a mixture of fatty acids which was saponified. From the soap by acidification was obtained 17.1 gm of fatty acids with an iodine value of 48.9. These acids were treated once more with 50 gm of mercuric acetate, 15 ml of acetic acid and 60 ml of methanol; after saponification of the reaction mixture and regeneration of the acids we obtained 8.3 gm of solid fatty acids with an iodine value of 84.5.

In order to remove the liquid unsaturated fatty acids still present in this fraction the mass of solid acids was subjected to a Twitchell-separation ('21), following which there remained a solid fraction which weighed 2.3 gm and had an iodine value of 81.4 and a melting point of 36°. The molecular weight of the fraction was about 280, but no further investigations were carried out in order to save the small fraction for biological testing. A comparison of the above data with those of Bertram suggests that the material obtained by us is

relatively pure although it is very likely that it contains traces of saturated fatty acids and oleic acid. We obtained 0.5% yield from the butter acids, a quantity considerably greater than that obtained by Bertram (about 0.01%).

The fraction obtained in this way, consisting chiefly of vaccenic acid, was tested by growth experiments in the same way as described above. In the first experiment 29 rats were used which had received the basal diet plus rape-seed oil during a preliminary period of 4 weeks, and then were divided into 3 groups. The 3 groups then received the basal diet plus 10% summer butter, the basal diet plus 10% rape-seed oil, and the basal diet plus 10% rape-seed oil to which the vaccenic acid fraction had been added, respectively. As the exact vaccenic acid content of the summer butter was unknown

TABLE 7

First growth test comparing summer butter, rape-seed oil, and rape-seed oil with vaccenic acid.

LITTER FROM WHICH RATS WERE TAKEN	GROUP I SUMMER BUTTER			GROUP II RAPE-SEED OIL			GROUP III RAPE-SEED OIL PLUS VACCENIC ACID FRACTION		
	Growth during successive periods:			Growth during successive periods:			Growth during successive periods:		
	4 weeks on rape-seed oil	1 week on butter	18 days on butter [1]	4 weeks on rape-seed oil	1 week on rape-seed oil	18 days on rape-seed oil [1]	4 weeks on rape-seed oil	1 week on rape-seed oil fraction	18 days on rape-seed oil fraction [1]
1	53	12	50	101	33	20	94	26	41
2	77	31	36	88	22	33			
3	90	34	39	87	32	37	93	29	53
4	100	29	50	91	24	34	90	18	46
6	74	29	63	67	19	35			
7	100	30	45	88	20	32	118	26	43
7	105	30	45						
8	85	18	46	83	19	34	70	20	40
9	68	24	33	67	22	33			
10				98	24	46	82	26	44
12				77	21	34	77	19	41
12							83	22	42
11				89	22	28	72	19	38
mean	84	26	45	85	23	33	87	23	43

[1] An analysis of variance for the figures for the last 18-day period shows that the probability of these results arising from random sampling is far below 1%.

the working assumption was made that it contained twice as much as we had actually isolated in our experiment. As much vaccenic acid as will then be contained in 20 gm of butter was added to the 10 gm of rape-seed oil mixed with each 90 gm of fat-free basal diet. The results of this experiment are given in table 7 where the data on growth during the preliminary period on rape-seed oil are given, followed by those

TABLE 8

Summary of experiments confirming tests reported in table 7. Group I — summer butter; group II — rape-seed oil; group III — rape-seed oil plus the vaccenic acid fraction.

SECOND COMPARISON				THIRD COMPARISON			
Litter from which rat was taken	Growth in final 16 days with group			Litter from which rat was taken	Growth in final 16 days with group		
	I	II	III		I	II	III
	gm	*gm*	*gm*		*gm*	*gm*	*gm*
4	62	13	50	1	51	46	54
6	39	35	32	2	42	42	45
7	51	39	48	4	43	34	40
7		29	48	7	55	38	44
9	47	27	37	8	45	43	43
9			36	11	44	42	48
10	54	48	44	12	47	32	44
12	33	34	35	14	57	39	
14	54	32	34	14		32	
3	33	23		15		38	49
11		44	53				
Mean	47	32	41	Mean	48	39	46
Analysis of variance: probability of chance occurrence 2%.				Analysis of variance: probability of chance occurring considerably less than 1%.			

for the growth exhibited during 2 periods upon the summer butter and vaccenic acid as well as the rape-seed oil. It is the figures for the growth during the last period to which we wish to direct attention. These data were subjected to an analysis of variance by the method of Snedecor ('46), and it was found that the probability of such results occurring purely on the basis of random sampling is considerably less than 1%. We may therefore regard the results as highly sig-

nificant and ascribe the responses obtained to the dietary treatment; that is, to the addition of vaccenic acid to the basal diet as compared with the addition of rape-seed oil and summer butter.

In order to confirm this result the same experiment was repeated twice with different groups of rats, the first experiment being continued for 2 weeks and the second experiment being continued for only 1 week. The results are shown in table 8. The probability of such results being obtained on the basis of pure chance is about 2% for the second experiment and well below 1% for experiment 3. They thus confirm exactly the first experiment reported.

Summary

1. The fraction obtained from summer butter fatty acids consisting chiefly of vaccenic acid has a marked growth-promoting action. When this fraction was added to a basal diet containing rape-seed oil growth became better; the difference in growth was highly significant.

2. The growth on summer butter itself was still better than the growth on the added quantity of vaccenic acid. The difference is significant but not highly significant. No reason can be ascribed for this difference since the exact quantity of vaccenic acid in the summer butter is not known.

SUMMARY AND CONCLUSION

Earlier experiments, in which we found a better growth in young albino rats on summer butterfat than on vegetable oils, were confirmed. The growth-promoting action was mainly present in the saponifiable fraction, that is, in the fatty acids. On fractionating the methyl esters of the acids of butterfat by distillation in vacuo, the activity was found in the C_{18}-fraction. On fractionating first with lead acetate and then with mercuric acetate we obtained a fraction chiefly containing vaccenic acid. This fraction possessed distinct growth-promoting qualities. It seems highly probable therefore that vaccenic acid is a growth-promoting factor present in summer butter.

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THE GROWTH-PROMOTING ACTION OF VACCENIC ACID

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As demonstrated in the preceding paper, a fraction obtained from the fatty acids of summer butter and consisting chiefly of vaccenic acid had, when added to a diet with rape-seed oil, a growth promoting action similar to that of summer butter. Vaccenic acid was thought to be responsible for the growth promoting action of summer butter. However, the possibility remained that other substances were present in the fraction, and that one of these was the cause of the growth promoting action of summer butter. To prove that vaccenic acid was the active principle it was desirable to obtain it from a source other than summer butter and show that it had the same effect. Therefore, we looked for a method to obtain vaccenic acid from other material.

Böeseken, Krimpen and Blanken ('30) described a method for hydrogenation of China wood oil, a fat consisting chiefly of alpha-oleostearic acid. This acid contains 3 double bonds. By modifying the method of Boeseken and his coworkers through use of a nickel-catalyst, and by partial hydrogenation it was possible to obtain a material consisting for the greater part of vaccenic acid, an acid with a single double bond between C_{11} and C_{12}. Vaccenic acid obtained in this manner has been shown to have the same effect as the growth promoting factor from summer butter. Though we have few experimental data to report, the results appear so striking that publication of the data seems desirable.

Twenty-two rats were placed on 3 different diets, the first containing 10% summer butter, the second 10% rape-seed oil, and the third 10% rape-seed oil to which 3% hydrogenated China wood oil was added (consequently 0.3% of the total food). The basal diet was the same as described in our earlier publications. The results are given in table 1.

TABLE 1

Individual growth of rats in 4 weeks.

LITTER	SUMMER BUTTER	RAPE-SEED OIL	RAPE-SEED OIL PLUS HYDROGENATED CHINA WOOD OIL
	gm	*gm*	*gm*
1	116	66	103
3	138	79	122
4	114	74	100
7		95	103
9	107	71	90
6	112	89	
8	109	89	
2		88	114
5		78	82
Mean growth:	116	81	102

Analysis of variance shows the probability of obtaining this result by pure chance to be far less than 1%.

We feel our experiments prove that China wood oil, added to a diet with rape-seed oil, has a growth promoting action. Because the fraction of summer butter and the hydrogenated wood oil have a similar growth promoting action, and since both fractions consist chiefly of vaccenic acid, we have concluded that the growth promoting factor of summer butter is vaccenic acid.

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ERRATA

The Journal of Nutrition, vol. 32, no. 6, December, 1946

Edwards, Leslie E. and others

Page 603, second line of second paragraph under *Histidine,* change 2 to 40 mg to read 2 to 40 μg.

The Journal of Nutrition, vol. 33, no. 1, January, 1947

Jukes, T. H., E. L. R. Stokstad and Margaret Belt

Page 3, line 4 from top should read —

"sodium pteroylglutamate 0.2 mg"